Building a hair cell

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Hair cells of the mammalian ear are responsible for the mechano-electrical transduction of sounds into electrical signals. They detect minute sound-induced vibrations of the cochlear partition via the displacement of their sensory hair bundles. Bundle displacements in the order of a few nanometers regulate ionic current through mechanically-gated channels located at the tips of the hairs (stereocilia). While inner hair cells (IHCs) are primarily responsible for relaying acoustic information to the central nervous system via afferent auditory nerve fibres, outer hair cells (OHCs) enhance the sensitivity and frequency selectivity of the cochlea by active mechanical amplification. Within the cochlea, hair cells are tonotopically organized such that mammals can perceive a wide range of sound frequencies. Moreover, IHCs are adapted for fast synaptic transmission through the development of specialized ribbon synapses, which are essential for encoding sound with accurate temporal precision. Such signalling fidelity is beyond the capabilities of most conventional synapses. The complexity of hair cell physiology, which varies as a function of cell type and location along the cochlea, requires them to mature through an extremely ordered progression of electrophysiological and morphological changes. In most adult rodents these changes occur over a period of three weeks from terminal mitosis at embryonic day 12-14 up to the onset of hearing at postnatal day 12 (P12). There are several critical check points on the way. Using various animal models, including mutant and knockout mice, we have identified several molecular mechanisms that are important for these check points.

The physiological differentiation of IHCs and OHCs depends on an intrinsic genetic programme coordinated by microRNA-96. In the absence of microRNA-96 hair cell differentiation is arrested at around birth in mice. In addition to microRNA-96, we discovered that the actin-binding protein Eps8 is required for several apparently unrelated aspects of IHC postnatal maturation. Although much of the development of hair cells depends on intrinsic genetic programmes, functional processes also shape the progress to maturity. This applies to the action potential activity that occurs during a critical period of differentiation. We found that action potentials are intrinsically generated by immature IHCs and that apical cells exhibit bursting activity as opposed to more sustained firing in basal cells. This difference in firing pattern along the cochlea could instruct the tonotopic differentiation of IHCs. The activity also influences the linearization of the exocytotic calcium dependence of the synaptic machinery in high-frequency post-hearing IHCs, which does not occur when the normal pattern of action potential activity is disrupted. We have recently demonstrated that this linearization depends on the expression of the synaptic protein synaptotagmin IV in adult IHCs. The final stage of IHC maturation includes a switch from Ca2+-dependent action potentials (APs) to graded receptor potentials driven by hair bundle displacement after P12. This switch is associated with a complete change in the IHC’s complement of ion channels, their synaptic biophysics and the reorganization of synaptic connections from and to IHCs. The above findings have allowed us to identify key molecules involved in the development of mammalian auditory hair cells and also to elucidate, at least in part, crucial physiological mechanisms that regulate hair cell differentiation.

Supported by the Royal Society, Wellcome Trust, RNID, Deafness Research UK, Physiological Society, Westfield Health and the University of Sheffield

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

100th Anniversary of the Anglo-American Expedition to Pikes Peak

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The 1911 Anglo-American Expedition to Pikes Peak was the most important high altitude expedition in the early 20th century. By way of introduction, it is useful to look at the state of high altitude physiology when the expedition was being organized. A watershed event had been the publication in 1878 of La Pression Barométrique by the eminent French physiologist, Paul Bert. He showed conclusively that the deleterious effects of high altitude could be attributed to the low partial pressure of oxygen. Previous theories had included bizarre explanations such as the failure of the reduced barometric pressure to press the head of the femur into the pelvis with the result that the required increased muscular effort caused fatigue. However Bert showed that the low PO2 was the culprit. Bert never carried out studies at high altitude and all his work was done in decompression chambers in the Sorbonne in Paris. However shortly after the publication of his book physiologists recognized the advantages of working at high altitude. In 1890 the first high altitude laboratory known as the Observatoire Vallot, was installed at an altitude of 4350 m on Mont Blanc. This was followed in 1893 by the Capanna Margherita which was erected on a peak of the Monte Rosa in Italy at an altitude of 4559 m as a result of the enthusiasm of the Italian physiologist Angelo Mosso. There was also an expedition to the Alta Vista Hut, altitude 3350 m in Tenerife, Canary Islands, in 1910. The principal figures of the Pikes Peak expedition were J.S. Haldane and C.C. Douglas from Oxford, Y. Henderson from Yale, and E.C. Schneider from Colorado Springs. The choice of Pikes Peak for the venue was largely determined by the difficult access of the stations on Mont Blanc and Monte Rosa and the primitive living conditions there. At the outset of planning the expedition, Haldane stated that he was looking for a “nice comfortable mountain” and, as Henderson related, Haldane thought that the rigors of working in the Capanna Margherita made it difficult to distinguish between the effects of the bad food and those of the high altitude. The same sentiment was aired by Joseph Barcroft who complained of the difficulties of gastronomy. One wonders if the British physiologists had the Right Stuff. However Pikes Peak had many advantages including its substantial altitude of 4300 m, easy access via a cog railway, comfortable, spacious living accommodation on the summit, and access to the nearby laboratory at Colorado College if additional equipment or supplies were needed. The classical design of the expedition included prior measurements at sea level in Oxford or New Haven, rapid ascent to 4300 m where the participants remained for five weeks, and then a rapid descent to sea level where further studies were made. An important feature was that many studies were made during the deacclimatization period. Few high altitude expeditions
have included these. The extensive scientific program included descriptions of acute mountain sickness, multiple measurements of alveolar gas and blood partial pressures before, during, and after the period at high altitude, changes of ventilation including the marked hyperpnea and periodic breathing, exercise studies, studies of blood hemoglobin and plasma volume, and limited cardiovascular measurements. A major interest was the relation between alveolar and arterial PO2, and the participants concluded that active secretion of oxygen by the lung was a feature of acclimatization although this was subsequently shown to be erroneous. The arterial PO2 was determined by an indirect method following carbon monoxide breathing and the precise reason for the errors is still not known. A colorful participant was Mabel FitzGerald who had collaborated with Haldane in Oxford several years previously. However she did not take part in the summit studies for reasons that are not completely clear. It has been alleged that this was because she was not chaperoned, but it is more likely that her presence would have complicated the living conditions. Instead of participating in the measurements on the summit she visited various Colorado mining camps accompanied only by a mule. In the process she collected extensive data on alveolar gas at different altitudes, and it is remarkable that her results are frequently cited even today because so few measurements have been made at relatively low altitudes. Mabel FitzGerald was able to join the Haldane centenary celebration in Oxford in 1961 at the age of 100 when she was given an MA degree and made a member of the Physiological Society. The Pikes Peak Expedition remains one of the most important, and its influence on subsequent high altitude studies was immense.

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**PL3**

**The Rhythm of Life: How your body clock makes you tick**

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An internal 24-hour biological clock (circadian clock) controls, modulates and fine-tunes our sleep patterns, alertness, mood, physical strength, blood pressure, and every other aspect of our physiology and behaviour. This ‘day within’ even changes our responses to drug treatments which can show large time-of-day effects.

Under normal conditions we experience a 24-hour pattern of light and dark, and our clock uses this signal to align biological time to the day and night. The clock is then capable of anticipating the differing demands of the 24-hour day and adjusting our biology in advance of the changing conditions. Body temperature drops, blood pressure decreases, tiredness increases in anticipation of going to bed. Whilst before dawn, metabolism is geared-up in anticipation of increased activity. The past decade has witnessed remarkable progress in understanding the mechanisms that generate circadian rhythms and sleep. We now know where in the brain the ‘master’ clock is located and how individual cells can generate molecular rhythms. We also know that the eye contains specialised light detectors, different from the cells that regulate vision, that detect the dawn/dusk signal.

In parallel with our increased understanding of mechanisms, there is a growing appreciation of the severe consequences of ignoring the impact of these rhythms on our physiology, health and quality of life. The presentation will consider how circadian rhythms are generated, regulated by light and why we can’t ignore our internal time in both medical treatments and in the way we organise our 24/7 society.

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**PL4**

**Moonlighting MHC1: Releasing the brake on synaptic plasticity**

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Connections in adult brain are highly precise, but they do not start out that way. Precision emerges during development as synaptic connections remodel, a process that requires neural activity (action potentials and synaptic transmission) and involves regression of some synapses and strengthening and stabilization of others. Neural activity also regulates neuronal genes. In an unbiased PCR-based differential screen, we made the completely unexpected discovery that MHC Class I genes are expressed in neurons and are upregulated by neural activity and visual experience (Corriveau et al, 1998; Goddard et al, 2007).

To assess requirements for MHC1 in the CNS, mutant mice that lack stable surface expression of all MHC1, or specific MHC1 genes, were examined. Synapse regression in the developing visual system did not occur, and in adult hippocampus synaptic strengthening was greater than normal (Huh et al, 2000; Datwani et al, 2009). These observations suggest that neuronal MHC1 may normally function in synaptic plasticity. Receptors could interact with neuronal MHC1 and carry out these activity-dependent synaptic processes. In a systematic search, mRNA for PirB, an innate immune receptor, was found highly expressed in neurons in many regions of mouse CNS. We generated mutant mice lacking PirB function and discovered that the extent of plasticity in visual cortex is increased (Syken et al., 2006), as is synaptic strengthening in the hippocampus. Thus, PirB, like MHC1, appears to function as a “brake” on synaptic plasticity in the CNS. Moreover, the commonality of phenotypes present in these mice suggests a model (Shatz, 2009) in which PirB may bind and transduce signals from MHC1 ligands in neurons.

Together, results imply that this family of molecules, thought previously to function only in the immune system, may also act at neuronal synapses to limit how much- or perhaps how quickly- synapse strength changes in response to new experience. These molecules may be crucial for controlling circuit excitability and stability in developing as well as adult brain, and changes in their function may contribute to developmental disorders such as Autism and Schizophrenia.


Regulation and functional significance of cardio-respiratory interactions in vertebrates

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Ventilation and cardiovascular function must be matched in all vertebrates to secure efficient delivery of oxygen to the respiring tissue as well as an adequate removal of waste products. The cardiorespiratory interaction is particularly evident in ectothermic vertebrates that typically exhibit a discontinuous breathing pattern with prolong apnoea where heart rate is very low and intermittent ventilatory bouts where heart rate may increase several-fold. The cardio-respiratory responses and their interactions are driven by central feed-forward mechanisms and afferent feed-back from peripheral receptors (1). The reflex roles of these receptors differ between fishes and air-breathing vertebrates. In fish, hypoxic stimulation of the branchial oxygen sensitive chemoreceptors that are homologues to the carotid chemoreceptors of mammals causes pronounced bradycardia and hyperventilation, while stimulation of O2 sensitive receptors and the resulting hyperventilation normally causes a tachycardia in air-breathing vertebrates (2,3). In amphibians and reptiles, the cardiorespiratory interactions also include selective changes in pulmonary perfusion achieved by changes in the cardiac shunt pattern (4). Taking an outset in the regulation of these responses, I will discuss a series of experiments attempting to reveal these functional correlates and potential selective value of the hypoxic bradycardia in fish and the cardiac shunt pattern (5,6). Finally, I will discuss how visceral organs appear to exert some cardiovascular control during digestion in reptiles, and possibly other vertebrates (7).


The author is supported by The Danish Research Council
Ischaemic preconditioning prevents the differentiation induced by ischaemia/reperfusion injury of rat cardiac fibroblasts into myofibroblasts

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Ischaemia/reperfusion (IR) injury is a key source of myocardial damage in humans following acute myocardial infarction, post-cardiac arrest, and heart transplantation (1). The beneficial effects of ischaemic preconditioning (IPC) in minimising IR-induced damage to cardiac muscle have been extensively studied, and involve activation of ATP-sensitive potassium (K<sub>ATP</sub>) channels (2). No previous studies have investigated the effects of IR injury or IPC in cardiac fibroblasts. Fibroblasts are responsible for maintaining the extracellular matrix in healthy hearts, and differentiate into the wound-healing phenotype, myofibroblasts, following cardiac injury (3). Our goals were to determine if: (a) IR injury induces cardiac fibroblasts to differentiate into myofibroblasts; and if so, (b) does IPC ameliorate the IR-induced myofibroblast differentiation? Hearts were removed from isoflurane-anaesthetised (5% induction, 2% maintenance; 1 l/min oxygen) adult female Sprague Dawley rats, and fibroblasts dissociated by standard enzymatic digestion (4). Freshly dissociated fibroblasts were exposed to 30, 60 or 120 minutes of ischaemia by coating pelleted cells with mineral oil (modified from 5), followed by 60 minutes of reperfusion when mineral oil was replaced with culture media. Fibroblasts were then placed under culture conditions and allowed to grow to ~70% confluency. Cultures were stained for expression of α-smooth muscle actin, a marker of myofibroblast differentiation (3). IR of all durations evoked significant differentiation of fibroblasts into myofibroblasts, with 88±1% (mean±S.E.M., n=5) of cells differentiating following 30 minutes of ischaemia, 93±2% (n=4) following 60 minutes of ischaemia, and 92±1% (n=5) following 120 minutes of ischaemia. In contrast, time controls indicated that only 37±2% (n=5, 30 min), 30±1% (n=5, 60 min), and 45±2% (n=4, 120 min.) of cells differentiated from fibroblasts into myofibroblasts without IR. IPC produced by 15 minutes of ischaemia and 30 minutes reperfusion, prior to 60 minutes of ischaemia and 60 minutes of reperfusion, significantly reduced the differentiation of fibroblasts into myofibroblasts from 88±2% (n=7, no IPC) to 46±4% (n=7, IPC) (Fig. 1). The beneficial effect of IPC was reduced if cells were treated with 10 μM glibenclamide, a K<sub>ATP</sub> channel blocker; in this protocol, 77±4% (n=8) of fibroblasts differentiated into myofibroblasts despite preconditioning (Fig. 1). These data indicate that IR injury strongly stimulates differentiation of cardiac fibroblasts into the wound-healing phenotype, the myofibroblast. IPC prevented IR-induced differentiation in a glibenclamide-sensitive manner, suggesting that activation of K<sub>ATP</sub> channels is part of the mechanism by which IPC protects cardiac fibroblasts.

Effect of ischaemia reperfusion injury, ischaemic preconditioning, and glibenclamide on rat cardiac fibroblast to myofibroblast differentiation. Following 60 minutes of ischaemia and 60 minutes of reperfusion, a significant percentage of fibroblasts were stimulated to differentiation into myofibroblasts, compared to time control. If cells were preconditioned with 15 minutes ischaemia and 30 minutes reperfusion prior to the 60 minutes ischaemia/60 minutes reperfusion, significantly fewer cells differentiated. Glibenclamide (10 μM) significantly limited the beneficial effect of IPC.


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Quantification of cardiomyocyte proliferation during early development and following heart laser injury in zebrafish embryo


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Adult Zebrafish (D.rerio) can regenerate heart ventricle after partial resection (Poss et al., 2002; Raya et al., 2003). We have developed a model of cardiac injury in the zebrafish embryo using a laser microbeam, which demonstrates the ability of the embryonic ventricle to recovery functionality after damage. The aim of this study is to assess cardiomyocyte loss and proliferation in this model. Tg(cmlc2:EGFP) zebrafish embryos underwent heart laser injury at 72 hours post fertilisation (hpf), control embryos received fin injury. Heart ventricle cardiomyocyte number (CMn) was assessed by counting DAPI stained nuclei in isolated hearts, at 48, 70 (pre-laser), 74 (post-laser), 96 and 120 hpf. Ejection fraction was measured by video image capture and diastolic-systolic area subtraction method using imageJ. In normal embryos, CMn increased by approximately 30% per day between 48hpf and 96hpf (138±16 vs 234±48, p<0.001) with a further increase by 58% between 96 and 120hpf (234±48 vs 369±34, p<0.001). Laser injury at 72 hours produced a small non-significant reduction in CMn by 15% compared with con-
trols (195±42 vs 219±45, p>0.05) but this recovered within 24 hours following laser injury (234±48 vs 252±21). Changes in CMn were mirrored by changes in ejection fraction with a significant fall after laser (20.4±0.7% vs 14.2±1.2%, p<0.001) and recovery by 24 hours (23.2±0.9% vs 23.4±1.4%, p=ns). Data are expressed as mean±SEM (n=5 per group; p=ANOVA) Cardiomyocyte proliferation occurs rapidly during development with acceleration of cell number on day 5. Loss and proliferation of cardiomyocytes contributes partially to the fall and subsequent functional recovery in this model of laser injury. Poss KD et al. (2002). Science 298, 2188–2190. Raya A et al. (2003). Proc Natl Acad Sci USA 100, 11889–11895. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C3

Using endothelial progenitor cells as a genetic shuttle to alter central control of cardiovascular function in conscious rats
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Emerging evidence indicates endothelial progenitor cell (EPC) dysfunction is a contributing factor in hypertension and as a result, EPCs could prove viable targets in its treatment 1. Our evidence shows endothelial dysfunction and inflammation of the brainstem microvasculature that includes regions responsible for regulating arterial pressure 2. Our aim is to use EPCs to target brainstem microvasculature in vivo and assess changes in central cardiovascular control. Bone marrow mononuclear cells from male Wistar rats (5% halothane until loss of pinch reflexes followed by cervical dislocation) were expanded in conditions appropriate for the endothelial lineage and confirmed subsequently 3-4. EPCs (60-90,000 cells) or control (Dulbecco’s Phosphate Buffered Saline vehicle without calcium and magnesium: DPBS, or differentiated rat brain endothelial cells: RBE4) were injected into dorsomedial medulla of normotensive (NT) and spontaneously hypertensive rats (SHR) following anaesthesia (5% halothane until loss of pinch reflex, then Ketamine (60mg/kg) and Domitor (250ug/kg)) given i.p. Following surgery Baytril (2.5%) antibiotic was given for 1 week in drinking water. EPCs were fluorescently tagged (CM-DiI) or transduced to express egFP(LV-hELF-1α-eGFP). EPCs were associated with the microvasculature, including arterioles as seen by immunofluorescence. Using radio-telemetry 5, we found chronic decreases in systolic pressure (SBP) and low frequency spectra of SBP (LF-SBP) relative to animals injected with DPBS or RBE4. Peak change in SBP in NT EPC (n=7) vs. NT DPBS (n=6) was -4.0±0.7 mmHg (p<0.001); NT EPC (n=7) vs. NT RBE4 (n=6) = -3.0±0.6 mmHg (p<0.001); no significant difference seen comparing NT RBE4 vs. NT DPBS. In SHR EPC (n=5) vs. SHR DPBS (n=6) there was a fall of -8±0.7 mmHg (p<0.001), which was greater than that seen in NT rats (p<0.001 two-way unpaired students t-test). In the EPC NT group there was a decrease in LF-SBP vs. DPBS -10±3% (p<0.01). In SHR there was a decrease in LF/HighFrequency pulse interval in the EPC group compared to DPBS group (-10±1% p<0.001), which may suggest a difference in mechanisms between NT and SHR EPC groups. In conclusion, EPCs injected into the parenchyma of the dorsomedial medulla associate with the microvasculature and alter cardiovascular autonomic function in both NT and SHR. The finding that both SBP and, indirectly, sympathetic activity decrease after EPC transplantation is consistent with pro-hypertensive effects of endothelial dysfunction within the brainstem. Our current work is transducing EPCs using lentiviral vector to express angiotensin converting enzyme-2 prior to administration to in vivo rats. In a pilot, we injected NT EPCs-eGFP into SHR (i.v.) and found a decrease in BP of ~20mmHg. Two-way paired students t-test used unless otherwise stated. Hadoke, P.W.F., 2010. Journal of Hypertension, 28:887–888 Paton, J.F.R., Waki, H., 2009. Neuroscience and Biobehavioral Reviews, 33:89–94 Caporali, A. et al., 2008. Circulation research, 103(2), e15-26 Fadini, G.P., et al., 2008. Atherosclerosis. 197:496–503 Waki, H. et al., 2006. Experimental Physiology, 201:213 MRC & Pfizer funded research.

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C4

Peripheral Cardiac Sympathetic dysfunction in the pre-hypertensive Spontaneously Hypertensive Rat
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Increased peripheral cardiac sympathetic activity has been identified in the spontaneously hypertensive rat (SHR) compared to age matched normotensive Wistar Kyoto (WKY) controls. However, it is not clear whether this sympathetic hyperactivity precedes the development of the hypertensive phenotype. We therefore compared peripheral cardiac sympathetic responsiveness in the SHR to the WKY at 3-6 week of age. Ventricular weight, body weight ratios and in-vivo measurements of mean arterial pressure via a 3F catheter inserted into the carotid artery under general anaesthesia (1-3% Isoflurane), demonstrated that SHRs (n=8) were without left ventricular hypertrophy and were normotensive at this age compared to WKys (n=7). In an isolated organ bath double atrial preparation with intact right stellate ganglion (at 37±0.5°C), there was a significantly increased (unpaired Students t-test, p<0.05) heart rate response to right stellate stimulation at 5 and 7Hz (15V, 1msec) in SHRs (n=9) compared to WKys (n=7). This was also associated with a significantly greater sensitivity to bath applied noradrenaline (100nM to 2µM) in SHRs (n=8) compared to WKys (n=9). After loading isolated atria with [3H]-noradrenaline (0.185MBq), there was a significantly greater release of [3H]-noradrenaline to field stimulation (5Hz, 15V, 1msec) in SHRs (n=9) compared to WKys (n=7). This was also associated with a significantly greater sensitivity to bath applied noradrenaline (100nM to 2µM) in SHRs (n=8) compared to WKys (n=9). After loading isolated atria with [3H]-noradrenaline (0.185MBq), there was a significantly greater release of [3H]-noradrenaline to field stimulation (5Hz, 15V, 1msec) in the SHR compared to the WKY (n=7 for both groups). Moreover, the presynaptic α2 receptor antagonist yohimbine (1µM), did not alter the [3H]-noradrenaline release between the two groups. In isolated cultured neurons from stellate ganglia, the magnitude of the calcium transient in response to depolarization from high extracellular potassium was significantly larger in SHRs (122.1±16.7%, n=10) compared to WKys (91.7±13.1%, n=8). These results show that the enhanced transient is also associated with increased cardiac noradrenaline release which translates into a greater heart rate responsiveness in young SHRs compared to WKYS. The
increased release of noradrenaline is likely due to a greater neuronal calcium transient rather than impaired presynaptic α2 receptor mediated autoinhibition. In conclusion, the autonomic phenotype in pre-hypertensive rats occurs both pre and post synthetically, and may represent an early marker in the development of hypertension itself.

This work was supported by an Academy of Medical Sciences/Wellcome Trust award and a British Heart Foundation Centre of Research Excellence award. NH is a Clinical Lecturer in Cardiovascular Medicine at the University of Oxford and Specialist Registrar in Cardiology at the Oxford Radcliffe Hospitals NHS Trust.

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C5

Synaptic profile of Nucleus Tractus Solitarius neurons involved with the peripheral chemoreflex pathways in rats


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The glomus cells in the carotid bodies (CB) detect low pO2 level in arterial blood and the carotid sinus nerve conveys this information to 2nd-order neurons in the nucleus tractus solitarius (NTS) via tractus solitarius (TS), which is part of the chemoreflex pathways. It has been demonstrated that in 2nd-order NTS neurons receiving inputs from the aortic depressor nerve (ADN), the TS stimulation presents high temporal fidelity. However, the temporal properties of synaptic activity in NTS neurons receiving inputs from CB are not well known. Herein using patch-clamp recordings combined with fluorescence labeling of chemosensitive afferents in NTS we studied TS evoked excitatory post-synaptic currents (TS-eEPSCs) on identified NTS 2nd-order neurons that receive afferent inputs from the CB in brainstem slices from Wistar rats (~300g). The experiment was performed in CB-NTS 2nd-order neurons recorded in the same experimental conditions. The amplitudes of TS-eEPSCs were similar in both groups but the latencies and standard deviation (SD) of latency (jitter) were significantly higher in the CB-NTS neurons (latency: 4.3 ± 0.2 ms; SD: 0.5 ± 0.04 ms, n=21) than in ADN-NTS neurons (latency: 3.4 ± 0.3 ms; SD: 0.2 ± 0.05 ms, n=13). The CB-NTS neurons also presented higher failure rate during high frequency trains of stimulus and in a complex series of double-labeling experiments we documented that some CB-NTS 2nd-order neurons send direct projections to the rostral-ventrolateral medulla (RVLM). We conclude that: a) CB-NTS 2nd-order neurons present temporally distinct post-synaptic currents when compared with ADN-NTS 2nd-order neurons; b) low SD of latency of TS-eEPSCs is not necessarily a characteristic of all 2nd-order neurons in the NTS and c) the presence of direct connections between these 2nd-order neurons in the NTS and RVLM is indicative that these synaptic properties of CB-NTS neurons are relevant for the processing of respiratory and autonomic responses to chemoreflex activation.

Supported by FAPESP and CNPQ

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C6

Does the dorsal motor nucleus of the vagus control cardiac chronotropism?

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Both the nucleus ambiguous (NA) and dorsal motor nucleus of the vagus (DMV) contain vagal preganglionic neurons with extensive projections to the cardiac vagal ganglia. While NA cardiac vagal preganglions exert a strong cardioinhibitory influence on heart rate, it is unclear whether a similar function exists for DMV preganglions. To test this, we used a viral vector-mediated optogenetic strategy to obtain repeatable neuronal specific activation and investigated the relative influence of NA and DMV neurons on cardiac chronotropy. Vagal preganglionic neurons were transduced with a lentiviral vector with a PRS8 promoter driving channelrhodopsin2 (ChR2) expression, and one week later were light-activated in the working heart-brainstem preparation (WHBP) (1), and heart rate responses recorded.

Male Wistar rats (P19, n=16) were injected with of Lenti-PRS8-Chr2-EYFP (1μl) into either the NA or DMV (left and/or right) under anaesthesia (ketamine 60mg.kg⁻¹/metadomide 25μg.kg⁻¹ i.p.). Subsequently, they were anaesthetised with Halothane (5%) (discontinued after decerebration) for surgical setup of the WHBP, and heart rate, perfusion pressure and phrenic nerve activity was monitored. Preganglionic neurons were light activated using a laser optrode (445nm) inserted over the DMV or NA, and heart rate recorded. In some cases a vagus nerve was recorded or atropine (10μg i.a.) was given. The brains were fixed (4% formalin), cut into 40μm coronal sections and injections sites identified using fluorescence microscopy. Values are mean ± S.E.M., compared by paired t-tests.

Optoactivation of NA or DMV neurons produced bradycardias that were titratable and temporally locked to the stimulus. Brisk responses were evoked by NA optoactivation (37±4 bpm, n=9, p<0.05), compared to modest DMV responses (-9±1 bpm, n=8, p>0.05) (NA vs DMV, p<0.05). Where both NA and DMV neurons were transduced in the same rat, a similar pattern was seen: strong NA with small DMV responses (-20 vs -4 bpm, respectively, n=1). Co-activation of DMV preganglions with vagal cardiorespiratory afferent evoked reflexes (eg peripheral chemoreflex) showed no evidence of potentiation of the bradycardia. Thoracic vagal recordings showed DMV and NA optoactivation was effective in evoking discharge and histology showed many more transduced cholinergic neurons in the DMV (>10x) than NA. All bradycardias were without change in phrenic nerve activity (p>0.05) or perfusion pressure (p>0.05), and were parasympathetically mediated as atropine abolished them (reduced by 95%). This study shows the feasibility of using optogenetics to manipulate cardiac parasympathetic drives and produce changes in end organ function. Our results have revealed a novel, albeit modest, cardio-inhibitory chronotropic role for the DMV. Further, activation of these neurons appears not to facilitate the peripheral chemoreceptor reflex.

Short term statin treatment does not affect blood pressure in 5 week old rats

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Statins (HMG Co-A reductase inhibitors) have been proven to be effective in secondary prevention of cardiovascular disease (CVD) with increasing evidence for their effectiveness in primary prevention (Ridker et al., 2008). In rats, maternal protein restriction leads to raised blood pressure and endothelial dysfunction in adult male offspring, which can be improved with long term chronic statin treatment (Torrens et al., 2009). The aim of the current study was to determine if statin treatment over an early two week period had any effect on blood pressure.

Female Wistar rats were fed either a control (C, 18 % casein) or protein restricted (PR, 9% casein) diet throughout gestation from conception to term. On delivery dams and pups were returned to standard chow. At 3 weeks of age pups were weaned and further divided into two subgroups of controls and those receiving statin. This gave the four experimental groups; control (C), control + statin (CS), protein restricted (PR) and PR + statin (PRS). At 5 weeks blood pressure was recorded using a non-invasive blood pressure monitor. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured and mean arterial pressure (MAP) was calculated. To minimise variations five stable readings were obtained, the highest and lowest of which were then discarded and the three remaining readings were used to calculate the mean. Results are expressed as mean ± SEM. Differences were assessed by one-way ANOVA. Significance was assumed at p<0.05.

Maternal weight gain, litter size and birth weight were similar between the groups. Neither systolic pressure (mmHg; C, 130.5±9.5, n=9; CS, 113.1±6.4, n=9; PR, 121.4±11.8, n=7; PRS, 119.3±6.6, n=7) nor diastolic pressure (mmHg; C, 93.3±9.0, n=9; CS, 78.6±6.0, n=9; PR, 89.3±9.2, n=7; PRS, 85.0±8.6, n=7) were different in male offspring. This was also true in female offspring for both systolic (mmHg; C, 117.2±11.3, n=7; CS, 121.2±9.7, n=8; PR, 126.4±9.7, n=7; PRS, 125.0±9.8, n=8) and diastolic pressure (mmHg; C, 82.8±10.5, n=7; CS, 86.2±8.5, n=8; PR, 91.7±9.7, n=7; PRS, 85.8±4.4, n=8).

The current data suggest that the blood pressure rises previously reported in this model are not present at five weeks of age. In addition, they show that even though long-term statin treatment has been shown to have positive effects in this model, they do not appear to be effective after a shorter exposure.

Ridker et al. (2008) NEJM 359:2195-2207

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Gestational diabetes impairs Nrf2/ARE mediated redox signalling in fetal endothelial cells: implications for developmental priming of endothelial dysfunction in offspring

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Gestational diabetes (GDM) affects ~7% pregnancies worldwide (Buchanan & Xiang, 2005), and offspring from gestational diabetic mothers are predisposed to type 2 diabetes and cardiovascular diseases in adulthood (Cheng et al., 2011). We previously reported abnormal NO production and reduced cell proliferation in foetal umbilical vein endothelial cells (HUVEC) cultured from GDM pregnancies (Sobrevia et al., 1995; Mann et al., 2003). HUVEC from normal and diabetic pregnancies were cultured in 20% serum M199 and then treated with the lipid peroxidation product 4-hydroxynonenal (HNE, 20μM) for 3-24h. In the present study, HNE induced adaptive increases in intracellular glutathione were diminished in GDM cells (GDM: 73 ± 5 vs normal: 112 ± 9 nmol/mg, mean ± S.E.M., n=5-7, p<0.01, Student’s t-test) and basal mitochondrial reactive oxygen species generation was elevated. HNE induced activation of the redox sensitive transcription factor NF-E2 related factor 2 (Nrf2) was impaired in GDM HUVEC, as evidenced by decreased mRNA expression of the glutamate/cystine transporter (xCT) and the phase II defence enzyme NAD(P)H quinone oxidoreductase 1 (NQO1). HNE induced DNA fragmentation was also increased in GDM compared to normal HUVEC, consistent with decreased endogenous antioxidant defences in GDM cells. A proteomic analysis further confirmed the altered phenotype of GDM HUVEC, characterised by markers of increased oxidative stress, reduced antioxidant protection and reduced proliferation. This altered vascular phenotype may contribute to an increased risk of type 2 diabetes and cardiovascular disease in the offspring of gestational diabetic mothers in later life.


Supported by BHF and China Scholarship Council

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Nitric oxide induces nuclear translocation of β-catenin in human umbilical vein endothelial cells

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Introduction: Endothelium-derived nitric oxide (NO), generated from l-arginine by the action of NO synthase type 3 (NOS-3), has been implicated in increased endothelial permeability through modulating β-catenin function in the adherens junc-
tion. Interaction between NOS-3 and associated proteins (e.g. heat shock protein-90, β-actin) is important in NOS-3 regulation. Here we investigated the hypotheses that endothelial cell NOS-3 directly interacts with β-catenin, and that NOS-3 activation with consequent endothelial NO generation results in β-catenin translocation to the nucleus, with resultant effects on gene transcription.

Methods and results: Human umbilical vein endothelial cells (HUVEC) were isolated and cultured to confluence at passage 3 from umbilical cords obtained with consent following healthy uncomplicated pregnancies. In HUVEC lysates, using co-immunoprecipitation, we found a direct association between NOS-3 and β-catenin, and the level of this association was increased in response to the NOS-3 agonists histamine (100μM), thrombin (1μM), salbutamol (1μM) and adenosine (100μM)). Immunocytochemistry revealed that, following NOS-3 activation, β-catenin translocated to the nucleus, and this was confirmed by western blotting of nuclear extracts for β-catenin. However, whereas histamine and thrombin (which activate NOS-3 in a Ca2+-sensitive manner) induced β-catenin nuclear translocation both in the absence and presence of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100μM), adenosine and salbutamol (which induce Ca2+-insensitive NOS-3 activation) did so in the absence but not the presence of L-NAME. The Ca2+ ionophore ionomycin similarly induced β-catenin nuclear translocation in an L-NAME-insensitive manner, as did the NO donor spermine NONOate (10 μM) and the stable cGMP analogue 8-bromo-cGMP (10 μM). Salbutamol-induced nuclear translocation of β-catenin was also inhibited by L-lysine (a competitive inhibitor of L-arginine transport; 1 mM) and ODQ (a specific inhibitor of soluble guanyl cyclase; 10 μM). Furthermore, in both HUVEC and HEK-293 cells, spermine NONOate, 8-bromo-cGMP and sildenafil (a phosphodiesterase type 5 inhibitor; 100nM) all resulted in T cell factor/β-catenin nuclear translocation in an L-NAME-insensitive manner, as did the NO donor spermine NONOate (10 μM) and the stable cGMP analogue 8-bromo-cGMP (10 μM). Salbutamol-induced nuclear translocation of β-catenin was also inhibited by L-lysine (a competitive inhibitor of L-arginine transport; 1 mM) and ODQ (a specific inhibitor of soluble guanyl cyclase; 10 μM). Furthermore, in both HUVEC and HEK-293 cells, spermine NONOate, 8-bromo-cGMP and sildenafil (a phosphodiesterase type 5 inhibitor; 100nM) all resulted in T cell factor/β-catenin-mediated transcriptional activation.

Conclusions: NOS-3 is associated with β-catenin in HUVEC, and this association increases in response to NOS-3 activation. Both NOS-3 stimulation or activation of the NO-cGMP pathway by other means induces nuclear translocation of β-catenin, with resultant transcriptional activation. This phenomenon may contribute importantly to the known effects of NO on gene transcriptional regulation.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C10

Maternal Dietary High Fat Impairs Vascular Function in Offspring

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Human populations exhibit temporal and cross-sectional variations in fatty acid intake. Variations in dietary saturated (SFA), polyunsaturated (PUFA) and trans fatty acid (TFA) intakes altered cardiovascular disease risk in humans1. Maternal high saturated fat diet during pregnancy and lactation in rats is associated with impairment of vascular function in offspring2, but it is not known whether differences in the type of maternal dietary fat influence future cardiovascular function in the offspring.

Female rats were fed either 7% (w/w) or 21% (w/w) safflower oil (SAO, enriched in linoleic acid), hydrogenated soybean oil (HSO, enriched in TFA), butter (enriched in SFA) or fish oil (FO, enriched in eicosapentaenoic and docosahexaenoic acids) from two weeks prior to mating until offspring were weaned at day 28 onto AIN93M (4% w/w soybean oil). Endothelial function (relaxation to acetylcholine; ACh, 0.1 nM to 1 μM) was measured ex vivo in thoracic aorta from 77 day old offspring by wire myography. Rat aorta relaxation to ACh is entirely dependent on nitric oxide3, so aortic tissue endothelial nitric oxide synthase (eNOS) mRNA expression was assessed by real-time RT-PCR. Data is % relaxation to ACh, mean ± SD, n = 6 per group. Statistical analysis was performed using a general linear model with Tukey’s post hoc testing, significance being ascribed at p <0.05.

There was a significant effect of sex (p = 0.027) and total maternal dietary fat (p = 0.0001), and an interactive effect of sex and maternal total dietary fat (p = 0.04) on ACh-induced relaxation in aorta, but there was no effect of type of maternal dietary fat. Relaxation to 30 nM ACh was impaired in male offspring of dams fed 21% SAO (0.5 ± 5.8), HSO (1.8 ± 15.5) or FO (6.1 ± 13.5), but not butter, compared to offspring of 7% dams (SAO 32.4 ± 18.5; HSO 37.6 ± 21.5; FO 46.4 ± 22.4). Relaxation was impaired in female offspring of dams fed 21% SAO (7.3 ± 10.7), butter (17.6 ± 10.3) or FO (7.6 ± 9.6), but not HSO, compared to offspring of 7% dams (SAO 35.9 ± 31.4; Butter 60.2 ± 23.8; FO 53.0 ± 16.0). eNOS expression was not significantly different between any groups.

The data show that maternal dietary high fat persistently impairs offspring aorta endothelial function, contingent on sex. This effect is not due to changes in eNOS expression.


This work was supported by the British Heart Foundation

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C11

Investigating the Physiology of Diabetic Pregnancies – Myometrial Contractility and the Effects of Insulin

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Worldwide, a significant number of diabetic pregnancies end up in caesarean sections (CS). We have examined whether insulin the main control for diabetes, may have an effect on the contractility of myometrium and the pathway of insulin action in the uterus. Methods - The effects of insulin (7pM-70nM) on spontaneous and oxytocin (1nM) stimulated contractility in myometrial strips from term diabetic and non diabetic women (with full consent) and 22day pregnant Wistar rat were examined. In some experiments intracellular Ca signals were simultaneously measured. Ouabain (Na pump inhibitor) or tetraethylammonium (K channel inhibitor) was added to contracting myometrium combined with insulin to
examine the mechanism of insulin's action. To examine the expression of IRβ immunohistochemistry and western blotting was used. Results: Insulin causes a dose-dependent decrease in myometrial contractions in pregnant rats and both diabetic and non-diabetic women, which was mirrored in the underlying calcium transients. In non-diabetes the decrease was significant (p<0.05) at concentrations of 70pM-700nM, whereas in diabetics the decrease was only significant at the higher concentrations of 70nM-700nM. At the highest concentration of insulin (700nM) the decrease in force in non-diabetes was significantly greater than diabetics (21.67±10.67% n=15, 42.20±9.73% n=10 respectively) relative to control period. In the presence of oxytocin, addition of insulin also decreased the force of myometrial contractility: in non-diabetes this was significant (p<0.05) for all concentrations, however the decreases were not significant in diabetics. The results of the immunohistochemistry showed a significant reduction (p=0.01) in the amount of IRβ expressed in the myometrium of diabetics compared to non-diabetics (13.9±1.6% (n=8), 21.4±1.8% (n=8)) respectively, which was confirmed by western blotting. When 10μM Ouabain was added to contracting myometrium in combination with increasing concentrations of insulin, the negative effect of insulin on contractility was inhibited for all concentrations. 5mM TEA also significantly inhibited the negative effect of insulin on myometrial contractility. Conclusions: Insulin causes a dose-dependent decrease in amplitude of spontaneous and oxytocin-stimulated contractions in human and rat myometrium. The decrease in calcium transients suggests that insulin is acting by decreasing calcium currents, and may partially stimulate the Na pump resulting in cell hyperpolarisation. The inhibitory effect of TEA on myometrial responses to insulin suggests insulin may also impede conductance of K+. The inhibitory effect of insulin on myometrial contractility was reduced in diabetics compared to non-diabetics. This may be due to the significant decrease in the expression of IRβ in the myometrium from diabetics compared to non-diabetics which we found.

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C12

Adrenomedullin 2 enhances macrovascular endothelial barrier function while it disrupts coronary microvascular barrier via differential regulation of Rac1

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Aims: Adrenomedullin 2 (ADM2) is a novel member of calcitonin-gene-related peptide (CGRP) family which acts via increased production of cAMP via calcitonin-receptor-like receptors (CRLR). Recently, it has been shown that ADM2 expression is enhanced in failing myocardium. The main objective of the present study was to analyse the effects and molecular mechanisms of ADM2 on macromolecule permeability of coronary microvascular and venular macrovascular endothelium.

Methods: In cultured rat coronary microvascular endothelial cells (RCEC) and human umbilical vein endothelial cells (HUVEC), the effects of ADM2 (10 nM) on monolayer permeability (albumin flux), RhoA, Rac1 and cdc42 activation (pull-down assay), actin cytoskeleton and VE-cadherin (confocal microscopy) translocation and contractile activation (myosin light chain phosphorylation; Western blotting) were analysed. Results: ADM2 increased permeability of RCEC and reduced permeability of HUVEC monolayers and increased intracellular cAMP concentrations in both cell types in a concentration-dependent manner. These IMD effects could be blocked by CGRP receptor inhibitor in RCEC while by adrenomedullin receptor 1 (AM1) in HUVEC monolayers. ADM2 caused a derangement of actin cytoskeleton accompanied by loss of VE-cadherin in RCEC, while it causes a rearrangement of actin cytoskeleton and VE-cadherin at cell-cell junctions in HUVEC monolayers. ADM2 inactivated RhoA/Rock pathway in both cell types; however, it inactivated Rac1 in RCEC, while activating Rac1 in HUVEC. Inhibition of RhoA/Rock pathway in RCEC but not in HUVEC resulted in inactivation of Rac1, derangement of actin cytoskeleton, and loss of barrier function. On the other hand activation of either RhoA or Rac1 in RCEC with specific activators rescued the ADM2-induced increased permeability of RCEC but not HUVEC monolayers.

Conclusion: The data of present study demonstrate that ADM2 has differential effect on permeability of RCEC and HUVEC. This differential effect of ADM2 is due to differential regulation of actin cytoskeleton dynamics and Rac1 activity. Moreover, Rac1 activity is regulated by RhoA/Rock pathway in RCEC but not in HUVEC. Furthermore, the study suggests that enhanced expression of ADM2 in failing myocardium could be deleterious factor leading to myocardial oedema.

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C13

Phospholipid activation of M-type potassium channels

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The primary membrane phospholipid involved in regulating M-type (Kv7.2/7.3) potassium channels is likely to be inositol-4,5-bisphosphate (PI(4,5)P2). Channels open on binding of PI(4,5)P2, while dissociation of PI(4,5)P2 [1,2], which may be caused by depletion of membrane PI(4,5)P2 following (e.g.) stimulation of muscarinic acetylcholine receptors, results in closure of Kv7.2/7.3 channels [1]. Although stimulation of Kv7.2/7.3 channels by PI(4,5)P2 is well established, the contribution of other membrane phospholipids has not been widely studied, but it is known that the homologous Kv7.1 protein can bind a range of anionic phospholipids [3]. The aim of this study was to compare activation of Kv7.2/7.3 channels stably expressed in Chinese Hamster Ovary cells by dioctanoyl (DiC8)-phosphoinositides and sphingosine phosphates applied in incremental concentrations via a fast microperfusion technique to isolated inside-out membrane patches held at -15 mV [4].

PI(4,5)P2 is an aliphatic compound which consists of a negatively charged hydrophilic inositol-1,4,5-trisphosphate (I(1,4,5)P3) head-group and a neutral hydrophobic diacylglycerol tail. PI(4)P, the precursor of PI(4,5)P2, and the product of its further phosphorylation, PI(3,4,5)P3, share conceptually similar structures. The effects of these phosphoinositides as well as I(1,4,5)P3 and I(4,5)P2 were compared with the effects of DiC8-PI(4,5)P2. The concentration-dependence of Kv7.2/7.3 channel activation was characterised by two component curves.
A novel viral tool to study ATP-mediated signalling in the brain

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For a long time astrocytes were seen as cells that only provide structural and metabolic support for neurons but this view is now changing rapidly. ATP has a pivotal role in the astrocyte-to-neurone communication and it is considered to be a major "gliotransmitter"1. Due to the lack of selective antagonists, studying ATP-mediated signalling in vivo and especially in chronic experiments is complicated. In order to overcome these obstacles, we have developed a viral vector encoding the Transmembrane Prostatic Acid Phosphatase (TMPAP)2. This lentiviral vector employs an elongation factor 1-alpha (EF1α) promoter to drive the expression of TMPAP, an enzyme that functions as an ecto-5'-nucleotidase. In order to visualise expression, TMPAP is fused to EGFP. The expression of TMPAP-EGFP was tested in primary astrocytes using a SP1 Leica confocal microscope. It was confirmed that the construct is correctly targeted to the plasma membrane. Characterization of the novel vector, LVV-SW-EGFP (1a-TMPAP-EGFP), was performed in primary cultures of rat astrocytes. As previously demonstrated when mechanically stimulated, astrocytes in cultures generate Ca2+ waves which propagate via spread of ATP. Cultures of astrocytes were prepared from the cerebral cortices, cerebellum and brainstem of 2-days-old (p2) Wistar rats and transduced with either LVV-SW-EF1a-TMPAP-EGFP or LVV-EF1a-EGFP which expressed EGFP as a control. Astrocytes were loaded with Rhod-2-AM and intracellular Ca2+ [Ca2+]i was monitored using a SP1 Leica confocal microscope at 34°C. To trigger a Ca2+ wave, astrocytes were mechanically stimulated with a patch pipette and changes in [Ca2+]i in the surrounding cells assessed. A line was drawn from the mechanically stimulated cell until each cell that responded and an average distance of Ca2+ wave spread was calculated. Mechanically stimulated TMPAP-EGFP expressing astrocytes showed a spread of Ca2+ wave of only 98 ± 6.6 μm (n=34) compared to EGFP expressing astrocytes where Ca2+ wave spread to 432 ± 40 μm (n=49), p<0.001, Unpaired t-Test. In a different experiment, astrocytes were loaded with Fura2-AM and mechanically stimulated as above. It was demonstrated that expression of TMPAP significantly shortened the duration of the stimulation-induced Ca2+ response at the site of stimulation. P2Y receptor antagonist MRS2179 (10 μM) also suppressed stimulation-induced Ca2+ waves, indicating that they are at least partially mediated by ATP. In summary, expression of TMPAP limits the spread of ATP-mediated signalling in cultured astrocytes, although it does not abolish it completely. LVV-SW-EF1a-TMPAP-EGFP is a novel tool to study purinergic signalling in the brain. It is currently used to investigate the role of ATP in cardio-respiratory control in vivo.

Gourine AV et al.(2010). Science 329(5991), 571
Zylka MJ et al.(2008). Neuron 60(1), 111

This work is funded by British Heart Foundation (BHF PG/08/009/24411).

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C14

VEGF-A165b is neuroprotective in in vitro models of diabetic and chemotherapy-induced sensory neuropathy and prevents ipsilateral DRG ATF-3 upregulation after nerve injury in vivo

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Alternative splicing of the Vascular Endothelial Growth Factor-A (VEGF-A) C-terminus results in the production of either pro-angiogenic (VEGF-Axxx) or anti-angiogenic (VEGF-Axxxb) isoforms (Bates et al., 2002). Over the last decade, the neuronal effects of VEGF-A have been recognised. VEGF-A165 promotes neurogenesis, axon extension and branching, and is neuroprotective in both the central and peripheral nervous systems. We previously identified a novel neuroprotective role for VEGF-A165b, the predominant VEGF-Axxxb isoform, for hippocampal neurones against L-glutamate-induced excitotoxicity, in vitro. Sensory neuronal damage and loss in diabetes and on chemotherapy lead to chronic pain and parasthesiae, severely affecting quality of life. We aimed to identify if VEGF-A165b is neuroprotective for peripheral sensory neurones in models of diabetic and chemotherapy-induced neuropathy, and in a traumatic nerve injury model.

Adult rat (male Wistar, ~250g) dorsal root ganglion neurones were removed, dissociated mechanically and with collagenase, and plated onto coverslips. 30μg/mL 5-fluoro-2'-deoxyuridine was added to inhibit glial proliferation. After overnight pre-incubation with ±2.5nM VEGF-A165b cells were treated with...
Integration strategies of pyramidal cells and Nkx2-1 interneurons in the early postnatal neocortex

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Our current view of the mechanisms that underlie the integration and consolidation of neural network is that promiscuous connections, initially supported by spontaneous activity intrinsic to the network are subsequently pruned and sculpted by emergent sensory input. To probe this further we have completed a series of studies using photostimulation to investigate what degree activity intrinsic to the neocortex is instructive in relation to pyramidal cell integration and whether or not interneuron populations with diverse mature characteristics adopt similar or divergent strategies. To map inputs we took advantage of laser scanning photostimulation (LSPS) in acute in vitro slices prepared from wildtype and Nkx2.1iCre;Z/EG mice. Mapping of pyramidal cells revealed that initial sources of input were diffuse, albeit slightly more concentrated from the immediate layer. Over time the input gradually got stronger and focused around the expected afferent input for the canonical circuit (Gilbert & Wiesel, 1983). In terms of layer 2/3 pyramidal cells this represented a relative reduction in layer 5 input (from 40.5% at P5-P8 to 26.4% at P17-P21; n=4), while perhaps more intriguingly in terms of layer 5 pyramidal cells it resulted in a pronounced shift toward later-born layer 2/3 input (increased from 27.1% input at P5-P8 to 45.9% at P17-P21). Using a modified LSPS protocol we were able to show that this change appears to be predicted by NMDA receptor-dependent inputs. Furthermore, analagous to the activation of silent synapses, evoking synchronous activity across cortical layers significantly enhanced input onto a subset of pyramidal cells prior to P12. Beyond this time point, input was immune to evoked activity in line with the fading of synchronised activity in vivo (Golshani et al., 2009) and the disappearance of NMDAR-only inputs observed using LSPS. To examine how locally-projecting GABAergic interneurons might assist in this process we recorded and mapped a genetically-defined population of Nkx2-1-derived interneurons over the corresponding timeframe. Our analysis revealed that there is a divergence in the strategies employed by fast-spiking (FS; n=19) and non-fast spiking/intrinsic bursting (NFS/IB; n=23) cells. The latter integrate early and do not develop further in terms of total synaptic input, although the layer distribution of afferent input is not static. In contrast FS cells which mediate lateral and feed-forward inhibition across all time points tested, bloom in terms of the scale of their input in parallel with the emergent pyramidal circuit. Thus it appears that layer 5 pyramidal cells and NFS/IB interneurons combine in the first few postnatal days to form a permissive network that acts to sculpt emergent connectivity intrinsic to the neocortex.


Research in the lab is funded by an HFSPO career development award to SJBB(CDA0023/2008).

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C16

Multi-channel electrophysiology suggests a functional laminar organisation within the superficial layer of the mouse superior colliculus

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Understanding how complex patterns of connectivity in the mammalian brain develop is the focus of considerable scientific activity, with the mouse visual system providing a versatile model system in this endeavour (Kim Hong et al, In-press1). We have undertaken an electrophysiological examination of the adult mouse superior colliculus to provide a functional context to such emergent anatomical circuit architecture. Wild type C57/BL6 adult male mice (7-16 weeks old), anesthetised and maintained using 1-1.3% halothane in oxygen/nitrous oxide (30:70), were artificially ventilated via a tracheotomy and their systemic physiology monitored and maintained (37±0.5°C, 3-5% end-tidal pCO2). In-house, three-dimensional in-vivo magnetic resonance imaging data determined a double-angle penetration protocol that enabled a 16 channel multi-electrode array to be inserted perpendicular to the surface of the superior colliculus with the overlying cortex left intact. This protocol enabled the 4 electrodes on each arm of the array to be introduced into the superior colliculus such that they were in register in visual space. The electrode separation on each arm of the array was 50μm, thus all 4 electrodes on each arm span the upper layer of the superior colliculus – stratum griseum superficiale (SGS). Sparse and sub-space noise together with sine-wave moving gratings presented from a bandwidth of spatial/temporal frequency space characterized the visual
physiological properties of the extra-cellular recordings. Multi-unit responses, local field potentials and automated spike thresholding, spike detection and clustering revealed single unit responses were all simultaneously employed. Our choice of electrode array with each arm separated by 125μm, availed separable receptive fields as determined by sparse noise presentations that were uniform in size (~5 degrees of visual space) irrespective of depth within the upper layer of the superior colliculus. We are in the process of characterizing more complex responses to our sub-space noise paradigms that putatively reveal more complex functional properties. Our findings using moving sine-wave gratings are however more readily apparent with phenotypic responses to the band-width of gratings evident at different depths within the SGS. Our findings suggest there is a functional laminar organization to upper visual layer of mouse superior colliculus (SGS). The results are however preliminary and subject to on-going characterization. While there have been significant recent advances in our understanding of the anatomical premise of such model circuits there is a significant gap in our knowledge linking their anatomy to function. The preliminary results to be presented provide an incremental framework to examine the functional consequences of normal and altered visual development.

Y. K. Hong, I. Kim and J. R. Sanes (In-press) ’Stereotyped axonal arbors of retinal ganglion cell subsets in the mouse superior colliculus’ The Journal of Comparative Neurology. DOI: 10.1002/cne.22595

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C18

Alpha band synchronisation in the reticular activating system and globus pallidus during anaesthesia-induced loss of consciousness in humans

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Introduction: The state of unconsciousness produced by general anaesthesia is associated with alpha frequency oscillations in the thalamus. Anaesthesia is further associated with a reduction in thalamic metabolic activity in imaging studies and this is also seen within the basal ganglia and reticular activating system (RAS). We therefore hypothesised that a similar alpha synchronisation occurs during induction of anaesthesia in the basal ganglia and RAS.

Methods: We recorded local field potentials (LFPs) from implanted deep brain electrodes during induction of anaesthesia in patients receiving therapy for dystonia or Parkinson’s disease. Electrodes were located in the globus pallidus interna of the basal ganglia and the pedunculopontine nucleus of the RAS. Anaesthesia was achieved using remifentanil and propofol. Indices of attainment of general anaesthesia were abolition of the blink reflex and fall in electroencephalographic bispectral index. LFPs were transformed into the frequency-power domain using fast Fourier Transform and the time-frequency domain using optimal spectral tracking (reference 1).

The dominant alpha frequency and its power was recorded and compared during twenty-second periods prior to admin-istration of anaesthetic agent and after attainment of anaesthesia.

Results: We studied 4 GPI and 5 PPN cases. Kolmogorov-Smirnov test demonstrated that the data was not normally-distributed and the Wilcoxon signed rank test was employed. Alpha (12Hz) power synchronisation was greater in all cases after anaesthesia onset compared to prior to anaesthetic administration (median power 41.314 μV2/Hz (SD+43.4 μV2/Hz) and 0.727 μV2/Hz (SD+3.4 μV2/Hz), respectively, where z=-2.666, df=9, p=0.008). There was a delay of alpha synchronisation of LFP power after administration of anaesthetic was initiated however the synchronisation was sudden and rapid when it occurred and always preceded loss of blink reflex.

Conclusions: Therefore, during anaesthesia-induced loss of consciousness, the RAS and GPI both became synchronised to the same alpha band reported in the thalamus. This continuous alpha activity located proximal to the thalamus supports the suggestion that it provides the electrical substrate for the blockade of inputs encoded at other frequencies from being transmitted via the ascending networks to the thalamus and, ultimately, the cortex.

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C19

Muscle afferent feedback contributions to ventilatory and cardiovascular control in humans

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The role of muscle metaboreceptive and mechanoreceptive afferents in human cardiorespiratory control can be examined using post exercise circulatory occlusion (PECO) and passive muscle stretch respectively avoiding the involvement of central command (Drew et al 2008).

In a series of trials, the respiratory and cardiovascular responses to combinations of passive calf muscle stretch and PECO during inhalation of a hypercapnic gas mixture were examined. These controlled for the effects of 1) the sensitising effects on muscle afferents of metabolites produced in exercise and 2) hypercapnia induced elevated central respiratory drive, in the absence of central command.

With local Ethical Committee approval, 12 healthy males performed 4 trials whilst positioned in a Biodex isokinetic dynamometer. Their right foot was attached to a footplate with the centre of rotation of their ankle aligned with that of Time-Frequency spectrum demonstrating onset of alpha power (12Hz) in the pedunculopontine nucleus of the reticular activating system. Line A depicts start of anaesthetic administration, B depicts loss of blink reflex.


Oxford Functional Neurosurgery is supported by grants from the Oxford Biomedical Research Centre of the NIHR and the The Centre of Excellence in Personalised Healthcare is funded by the Wellcome Trust and EPSRC under grant number WT088877/Z/09/Z.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
the machine. During the test phases of each trial, circulation through the right leg was occluded by inflation of a thigh cuff to 200mmHg. Subsequently subjects either rested for 1.5 min, Control Trial (Con) or plantarflexed their ankle at 50% maximum force for 1.5 min. Exercise Trial (Ex). Thereafter in each trial a further 7.5 min of occlusion followed which comprised a 3.5 min rest period, a 3 min sustained calf stretch period (performed automatically by the Biodex) and a further 1 min rest period post stretch. These two trials were performed breathing air and were repeated breathing a normoxic, hypercapnic (5% CO₂) gas mixture (CO₂ trial and CO₂+Ex trial). Minute ventilation (VE), mean arterial pressure (MAP) and heart rate (HR) were continuously recorded.

Inhalation of the hypercapnic gas increased baseline VE, HR and MAP (+26.5±2.3 L.min⁻¹, +6.7±2.5 beats.min⁻¹, +9.7±3.3 mmHg; Mean±S.E.M.) above control values (9.0±0.7 L.min⁻¹, 63±2.5 beats.min⁻¹, 87.6±2.6 mmHg) respectively. Exercise caused a further significant increase in these variables from baseline during both trials (P<0.05) ANOVA. MAP remained significantly elevated above baseline during PECO (P<0.05).

VE returned to baseline levels during PECO in the Ex trial but remained at end exercise levels (+7.6±1.9 L.min⁻¹) during PECO in the CO₂+Ex trial. Passive stretch caused a 2.2±0.6 L.min⁻¹ further increase in VE during the CO₂+Ex trial and a 3.3±1.3 L.min⁻¹ increase in the CO₂ trial. Stretch caused no increase in VE when participants inhaled air.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C20

The influence of systemic sympathetic activity on cerebral blood flow and cerebrovascular reactivity at rest and during exercise: Alterations with ageing

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Cerebral blood flow (CBF) is regulated by several factors, including arterial PCO₂ and arterial blood pressure (BP). To a lesser extent, CBF may also be influenced directly or indirectly by changes in cardiac output and sympathetic nerve activity (SNA). Given that SNA is known to be an important regulator of MAP via its effect on peripheral vascular resistance and cardiac output, we examined the effect of age on the influence of systemic sympathetic activity on regulating cerebral perfusion at rest and during exercise.

Eight younger (aged: 26±5 y) and eight older (aged: 56±3 y) participants were tested at supine rest and during a 10-min exercise test (at 50% heart rate range) before (control) and 90 min following the administration of selective α₁-blockade (Prazosin; 1 mg/20 kg body weight). Continuous measures of middle cerebral artery blood flow velocity (MCAv, Transcranial Doppler); MAP (intra-arterial and/or Finometer); heart rate (HR, ECG) and end-tidal PCO₂ (PETCO₂) were obtained during steady-state eupcapnia (room air) and hypercapnia (7% CO₂; 93% O₂) conditions at rest and during exercise.

At supine rest, α₁-adrenoreceptor blockade decreased MAP by a comparable extent in both younger (82 to 68 mmHg) and older (97 to 80 mmHg) participants. In the control condition, exercise-induced MAP increases were similar for both groups (Δ17±24 mmHg; P=0.45). Following blockade, exercise-induced increases were abolished in the young (Δ5 mmHg; P=0.24 vs. rest) yet remained in the old (Δ15 mmHg; P<0.01 vs. rest). Following blockade, MCAv was reduced (P<0.01) for both young and old at rest (76 to 65 cm/s) (young) and 60 to 48 cm/s (old)). Exercise-induced increases in MCAv were abolished in the young following blockade (Δ18 vs. 1 cm/s; interaction effect: P<0.01), whilst the older group’s response was unaffected (Δ5 vs. 2 cm/s; interaction effect: P=0.66). Unexpectedly, CBF responsiveness to hypercapnia was reduced in the young during exercise (3.1 to 2.2 cm/s/mMgHg), whilst the older group showed the expected increase (2.3 to 3.0 cm/s/mMgHg). Following blockade, hypercapnia CBF responsiveness increased for both groups at rest and during exercise (P<0.05). The reliance on α₁-sympathetically driven vasoconstriction during exercise is critical in the young but seems to be a redundant mechanism in order to maintain MAP with advancing age. Regardless of the pathway, exercise-induced elevations in MAP with ageing would seem beneficial in the regulation of MCAv. Furthermore, these data provide evidence for sympathetically mediated constraint on CBF-CO₂ responsiveness during rest and exercise.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C21

Cerebral blood flow in heart transplant recipients: rest and during exercise

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Background and hypotheses: Pathological impairments in cardiac output may impact on cerebral blood flow velocity (CBFv). Prior studies that have measured CBF in heart transplant recipients (HTR) has reported increases 25-53% 1-6 months post-transplant. It is unknown if CBFv is altered over the longer-term (i.e. years) following transplant or during progressive exercise stress. The aim of this study was to examine the influence of long-term heart transplantation on the regulation of CBFv at rest and during progressive exercise. To address this aim, we used stable HTR who have a reported inability to acutely increase cardiac output during exercise. Two hypotheses were tested: 1) CBFv would be similar in HTR when compared to age-matched controls (AM), but lower than young controls (YC); 2) that during exercise, the HTR would have reduced elevations in CBFv compared with AM and YC.

Methods: 7 male clinically stable HTR (62 ± 9 yrs of age and 9 ± 7 yrs post-transplant), 7 male AM (62 ± 7 yrs), and 7 male
YC (22 ± 3 yrs) were recruited for this study. Bilateral middle cerebral arterial trees were sonicated transcranial Doppler ultrasound to obtain an index of CBFV. Data were obtained while seated (rest) and during a progressive cycling test to volitional exhaustion. A repeated measures ANOVA was applied to identify differences across exercise intensity. Comparisons between groups were performed with Fischer’s LSD post hoc test.

Results: Table 1 provides an overview of the cerebrovascular, cardiovascular, and cardiorespiratory responses at rest and during the progressive exercise test.

Conclusion: Following long-term HTR, CBFV are comparable to AM counterparts; therefore, at rest it is the age of the brain and its cerebral vasculature that affects CBFV more so than the younger donor heart. Moreover, during progressive exercise, CBFV is well maintained in HTR.

Table 1. Summary of cerebrovascular, cardiovascular, and cardiorespiratory responses

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Group</th>
<th>MCAmean (cm/s)</th>
<th>HReserve (mmHg)</th>
<th>APBmean (mmHg)</th>
<th>PUT CBF (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>HTR</td>
<td>40 ± 12</td>
<td>0 ± 0</td>
<td>99 ± 4</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Rest</td>
<td>AM</td>
<td>33 ± 7</td>
<td>0 ± 0</td>
<td>106 ± 19</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>Rest</td>
<td>YC</td>
<td>60 ± 94</td>
<td>0 ± 0</td>
<td>91 ± 4</td>
<td>37 ± 4.41</td>
</tr>
<tr>
<td>5% VO2peak</td>
<td>HTR</td>
<td>45 ± 10</td>
<td>14 ± 5</td>
<td>107 ± 4</td>
<td>35 ± 3.3</td>
</tr>
<tr>
<td>5% VO2peak</td>
<td>AM</td>
<td>52 ± 10</td>
<td>26 ± 9</td>
<td>119 ± 12</td>
<td>37 ± 4.3</td>
</tr>
<tr>
<td>5% VO2peak</td>
<td>YC</td>
<td>84 ± 171</td>
<td>51 ± 71</td>
<td>99 ± 6</td>
<td>43 ± 14.4</td>
</tr>
<tr>
<td>7% VO2peak</td>
<td>HTR</td>
<td>47 ± 11</td>
<td>32 ± 13</td>
<td>114 ± 13</td>
<td>34 ± 2.1</td>
</tr>
<tr>
<td>7% VO2peak</td>
<td>AM</td>
<td>54 ± 8</td>
<td>58 ± 28</td>
<td>120 ± 15</td>
<td>37 ± 2.7</td>
</tr>
<tr>
<td>7% VO2peak</td>
<td>YC</td>
<td>84 ± 171</td>
<td>51 ± 71</td>
<td>99 ± 6</td>
<td>43 ± 14.4</td>
</tr>
<tr>
<td>9% VO2peak</td>
<td>HTR</td>
<td>51 ± 18</td>
<td>69 ± 28</td>
<td>137 ± 17</td>
<td>35 ± 5.5</td>
</tr>
<tr>
<td>9% VO2peak</td>
<td>AM</td>
<td>54 ± 6</td>
<td>69 ± 28</td>
<td>137 ± 17</td>
<td>35 ± 5.5</td>
</tr>
<tr>
<td>9% VO2peak</td>
<td>YC</td>
<td>75 ± 6</td>
<td>133 ± 15</td>
<td>180 ± 74</td>
<td>39 ± 1.5</td>
</tr>
<tr>
<td>10% VO2peak</td>
<td>HTR</td>
<td>42 ± 11</td>
<td>60 ± 15</td>
<td>138 ± 21</td>
<td>36 ± 5.5</td>
</tr>
<tr>
<td>10% VO2peak</td>
<td>AM</td>
<td>53 ± 8</td>
<td>80 ± 26</td>
<td>129 ± 13</td>
<td>32 ± 4.8</td>
</tr>
<tr>
<td>100% VO2peak</td>
<td>HTR</td>
<td>65 ± 99</td>
<td>124 ± 127</td>
<td>311 ± 271</td>
<td>28 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SD. * denotes significance (P<0.05) between HTR-AM, †denotes significance between HTR-YC, ‡denotes significance between AM-YC.


Pericellular Ca²⁺ recycling potentiates thrombin-evoked Ca²⁺ signals in human platelets

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Previous work has demonstrated a key role for the Na⁺/Ca²⁺ exchanger (NCX) in modulating store-operated calcium entry, as well as collagen- and P2X1-evoked Ca²⁺ signals in human platelets [1-3]. Initial studies examining the effects of the NCX inhibitors SN-6 and KB-R7943 on thrombin-evoked Ca²⁺ signals in the absence of extracellular Ca²⁺ demonstrated a seemingly paradoxical inhibition of the thrombin-evoked rise in cytosolic [Ca²⁺]. We have now investigated this further by examining the effects of NCX inhibitors on Ca²⁺ fluxes in different compartments in and around human platelets. Thrombin-evoked Ca²⁺ signals in the cytosol or pericellular space were monitored in Fura-2- or FFP-18-loaded cells respectively. Changes in the extracellular Ca²⁺ concentration were monitored using Fluo-4 K⁺ salt. Platelets were stimulated with 0.5 U/ml thrombin in the presence of 1 mM EGTA. Data are presented as mean ± SEM. Statistical significance was assessed using Student’s t-test. KB-R7943 (50 μM) or SN-6 (50 μM) reduced thrombin-evoked rises in cytosolic (48.9 ± 8.9% and 65.0 ± 5.3% of control respectively; both p < 0.05; n = 5), extracellular (14.9 ± 2.8% and 77.4 ± 9.7% of control respectively; p < 0.05; n = 6), and pericellular (signal abolished and 41.8 ± 6.6% of control respectively; p < 0.05; n = 5) Ca²⁺ concentration. To investigate whether the pericellular Ca²⁺ signal influenced the cytosolic Ca²⁺ signal we replaced in the extracellular medium the slow Ca²⁺ chelator, EGTA, with same concentration of the faster Ca²⁺ chelator, BAPTA. This reduced the thrombin-evoked rises in pericellular (54.5 ± 9.8% of control; p < 0.05; n = 7) and cytosolic (72.8 ± 1.9% of control; p < 0.05; n = 6) Ca²⁺ concentration. Furthermore, blocking plasma membrane Ca²⁺ channels using MRS-1845 (30 μM) and 5'-loido-resiniferatoxin (20 μM) also reduced the cytosolic Ca²⁺ signal evoked by thrombin in the absence of extracellular Ca²⁺ (54.4 ± 3.0% of control; p < 0.05; n = 7). These results suggested that the pericellular Ca²⁺ signal might influence Ca²⁺ dynamics in the cytosol through Ca²⁺ recycling back into the cell across the plasma membrane. We therefore attempted to visualize the pericellular Ca²⁺ signal by imaging thrombin-activated single platelets immobilized on fibrinogen-coated slides in a medium containing Fluo-4 K⁺ salt. We observed pericellular Ca²⁺ signals contained within the boundary of the cells that spread in a slow, directionally-restricted manner. These results suggest that Ca²⁺ is specifically removed from the cytosol into invaginations of the platelet plasma membrane known as the open canalicular system (OCS), that NCXs create a rise in [Ca²⁺] in the OCS of thrombin-stimulated platelets and that Ca²⁺ can recycle from the OCS back into the cytosol so helping to maintain the rise in cytosolic Ca²⁺ concentration.


Supported by the British Heart Foundation.
Exercise-induced oxidative-nitrosative stress is associated with impaired dynamic cerebral autoregulation and blood-brain barrier integrity

D.M. Bailey1,2, K.A. Evans1, J. McEneny3, I.S. Young3, D.A. Hullin4, P.E. James5, S. Ogoh6, P.N. Ainslie7, M. Culcasi2, S. Pietri2 and D. Janigro8

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Background and hypothesis: Cerebral autoregulation (CA) is a homeostatic mechanism that serves to maintain cerebral blood flow (CBF) constant over a wide range of perfusion pressures. It is especially important for the human brain given its reliance on oxygen and glucose to support the metabolic demands of neuronal activity and need to protect tissue from hypoxic/hyper-perfusion (Willie et al., in-the-press). In rodents, subdural perfusion with the superoxide anion impaired the dynamic range of CA (dCA) (Zagorac et al., 2005) though human data are lacking. Given that acute exercise is an established stimulus for oxidative-nitrosative (OX-NOX) stress (Bailey et al., 2010), the current study examined if intense exercise would increase blood brain-barrier (BBB) permeability subsequent to impaired dCA.

Methods: Eight healthy males were examined at rest and after an incremental bout of semi-recumbent cycling exercise to exhaustion. Changes in a dCA index [ARI (Tiecks et al., 1995)] were determined during the recovery period from continuous recordings of blood flow velocity in the middle cerebral artery (MCAv, trans-cranial Doppler ultrasound) and mean arterial pressure (finger photoplethysmography) during transiently induced hypotenison (Aaslid et al., 2010). Electron paramagnetic resonance spectroscopy combined with ex-vivo spin trapping and ozone-based chemiluminescence were employed for direct detection of spin-trapped free radicals and nitric oxide (NO) respectively in venous blood. Neuron-specific enolase (NSE), S100β, and 3-nitrotyrosine (3-NT) were determined by Enzyme-Linked Immuno-Sorbert Assay. Following confirmation of distribution normality using Shapiro-Wilk W-tests, data were analysed using paired samples t-tests and relationships determined with Pearson Product Moment Correlations. Significance was established at P ≤ 0.05 and data expressed as mean ± standard deviation (SD).

Results: While exercise did not alter MCAv (rest: 49 ± 6 vs. 47 ± 8 cm/sec, P > 0.05), it caused a mild reduction in ARI [6.9 ± 0.6 arbitrary units (AU) to 5.5 ± 0.9 AU, P < 0.05]. This reduction correlated directly against the exercise-induced increase in the ascorbate radical (global free radical flux), 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide and α-phenyl-tert-butylnitrone-adducts (identified through simulation as a mixture of primary hydroxyl and secondary alkoxy-alkyl radicals), 3-NT and S100β (r = -0.66 to -0.81, P ≤ 0.05). In contrast, no changes in NSE or (total) NO were observed.

Conclusion: These findings are the first to suggest that intense exercise has the potential to disrupt the BBB without causing structural brain damage subsequent to a free radical-mediated impairment in dCA.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C23

Exercise-induced oxidative-nitrosative stress is associated with impaired dynamic cerebral autoregulation and blood-brain barrier integrity

D.M. Bailey1,2, K.A. Evans1, J. McEneny3, I.S. Young3, D.A. Hullin4, P.E. James5, S. Ogoh6, P.N. Ainslie7, M. Culcasi2, S. Pietri2 and D. Janigro8

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Methods: Eight healthy males were examined at rest and after an incremental bout of semi-recumbent cycling exercise to exhaustion. Changes in a dCA index [ARI (Tiecks et al., 1995)] were determined during the recovery period from continuous recordings of blood flow velocity in the middle cerebral artery (MCAv, trans-cranial Doppler ultrasound) and mean arterial pressure (finger photoplethysmography) during transiently induced hypotenison (Aaslid et al., 2010). Electron paramagnetic resonance spectroscopy combined with ex-vivo spin trapping and ozone-based chemiluminescence were employed for direct detection of spin-trapped free radicals and nitric oxide (NO) respectively in venous blood. Neuron-specific enolase (NSE), S100β, and 3-nitrotyrosine (3-NT) were determined by Enzyme-Linked Immuno-Sorbert Assay. Following confirmation of distribution normality using Shapiro-Wilk W-tests, data were analysed using paired samples t-tests and relationships determined with Pearson Product Moment Correlations. Significance was established at P ≤ 0.05 and data expressed as mean ± standard deviation (SD).

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Conclusion: These findings are the first to suggest that intense exercise has the potential to disrupt the BBB without causing structural brain damage subsequent to a free radical-mediated impairment in dCA.

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C23

Exercise increases endothelial nitric oxide synthase phosphorylation in the microvasculature and sarcolemma of human skeletal muscle

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Nitric oxide (NO) plays a multifaceted role in the physiological responses and adaptive processes during and following exercise within the skeletal muscle and its microvasculature. Endothelial nitric oxide synthase (eNOS) is one of the three NOS isoforms responsible for synthesis of NO. eNOS is expressed in both vascular endothelial cells and skeletal muscle and therefore changes in its activity may be responsible for the increase in NO production during exercise. The aim of the current study was to assess eNOS serine1177 phosphorylation in response to acute exercise, using quantitative immunofluorescence microscopy both in the skeletal muscle sarcolemma and microvascular endothelium in humans.

Eight young sedentary males (age 21 ± 2 years, BMI 23 ± 1 kg/m², VO2peak 42 ± 8 ml/kg/min) completed one hour of cycling at approximately 65% VO2peak. Muscle biopsies (1% lignocaine as local anaesthesia) from the vastus lateralis were taken prior to, and immediately after exercise. eNOS serine1177 phosphorylation was assessed in the skeletal muscle microvascular endothelium and sarcolemma independently, using quantitative immunofluorescence microscopy. eNOS serine1177 phosphorylation was displayed as fold change from rest ± standard error.

eNOS serine1177 staining was present in the microvessels in both resting and post exercise images, and colocalised with the endothelial marker UEA1-FITC. eNOS serine1177 staining was also present at the sarcolemma in both resting and post exercise images, and colocalised with the membrane marker wheat germ agglutinin-350. Exercise increased endothelial eNOS serine1177 phosphorylation by 25% (1.25 ± 0.08 fold change from resting, P < 0.05). Exercise also increased sarcolemmal eNOS serine1177 phosphorylation by 14% (1.14 ± 0.05 fold change from rest, P < 0.05).

We have shown that eNOS serine1177 phosphorylation is increased following exercise in both the sarcolemma and
microvascular endothelium in skeletal muscle. These results suggest that eNOS is at least partially responsible for NO production in both the microvasculature and sarcolemma during exercise. Quantitative immunofluorescence microscopy allowed for the independent analysis of eNOS phosphorylation in the microvasculature and sarcolemma of skeletal muscle. This novel approach may improve our understanding of the mechanisms and location of increased NO production within the skeletal muscle in response to exercise.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Identification of Calcium-Sensing Receptor$$^{S875}$$ as a novel phosphorylation site contributing to the feedback regulation of receptor activity

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Extracellular calcium (Ca$$^{2+}$$) homeostasis is controlled by the action of the Ca$$^{2+}$$-sensing receptor (CaR) on parathyroid hormone secretion and renal Ca$$^{2+}$$ reabsorption. CaR contains 5 intracellular serine/threonine residues predicted to be protein kinase C (PKC) phosphorylation sequences. Previously we have shown that one of these, CaR$$^{T876}$$, represents the key phosphorylation site responsible for protein kinase C-mediated inhibition of CaR-elicited Ca$$^{2+}$$ mobilisation in vitro [1,2] and PTH secretion in vivo [3]. Importantly though, we found that CaR$$^{T876}$$ cannot be the sole determinant of the PKC response and previous work suggests that the other 4 known PKC sites play little or no role [4]. For the structurally homologous metabotropic glutamate receptor (mGlur) 5, the equivalent PKC site is Ser-839 which aligns in CaR not with CaR$$^{T888}$$ but with CaR$$^{S875}$$, which has not been considered a putative PKC site before.

Thus, here we examined the effect on CaR responsiveness of mutating CaR$$^{S875}$$ to alanine (non-phosphorylatable) or asparagine (phosphomimetic) in either wild-type (WT) CaR or a CaR$$^{S875A}$$ mutant (QuickChange) expressed in Fura2-loaded HEK 293 cells. For CaR$$^{S875A}$$, the Ca$$^{2+}$$-concentration dependence was significantly left-shifted (EC$$^{50}$$, 2.7±0.1 vs. WT, 3.4±0.1 mM; P<0.05 by ANOVA, N=6) while for CaR$$^{S875D}$$, the relationship was shifted to the right (4.2±0.3 vs. WT, 3.4±0.1 mM; P<0.05). Thus, CaR$$^{S875}$$ most likely represents a phosphorylation site similar to CaR$$^{T876}$$ (EC$$^{50}$$ for CaR$$^{S875A}$$ also 2.7±0.1). Next, we showed that the Ca$$^{2+}$$-responsiveness of the double mutant CaR$$^{S75A,T888A}$$ was left-shifted to an even greater extent than for CaR$$^{T888}$$ alone (EC$$^{50}$$, 2.2±0.1 vs. 2.7±0.1 respectively; P<0.01) demonstrating the additive effects of the two mutations.

Next, in mGlur5 the neighbouring threonine-840 residue is permissive for mGlur5$$^{S839}$$ phosphorylation and thus we also investigated the responsiveness of the mutants CaR$$^{T876A}$$ and CaR$$^{S875D}$$ For CaR$$^{T876A}$$, the Ca$$^{2+}$$-concentration dependence was no different from WT (EC$$^{50}$$, 3.8±0.4 vs. WT, 3.4±0.1 mM) whereas for CaR$$^{S875D}$$, the relationship was shifted markedly to the right (4.9±0.3: P<0.05 vs. WT). This suggests that CaR$$^{S875}$$ is not normally a phosphorylation site but that its phosphomimetic mutation is also inhibitory for CaR-mediated Ca$$^{2+}$$i mobilisation.

Therefore, CaR$$^{S875}$$ is most likely a previously unrecognised PKC phosphorylation site that, together with CaR$$^{T888}$$, acts to shape CaR signalling and maintain the CaR-mediated control of Ca$$^{2+}$$i homeostasis.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Pepsinogen is nitrated in the stomach in vivo: a dual function for dietary nitrate with functional and physiological consequences

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Dietary nitrate may be a novel regulator of physiological functions in the gastrointestinal tract, due to its stepwise reduc-
tion to nitrite and nitric oxide (NO). While NO is engaged in
gastroprotection, nitrite generates nitrogen oxides able to
nurate tyrosine residues of endogenous proteins. This work
studies the impact of nitrite dietary supplementation in the
nitration status of the healthy and ulcerated rat gastric mucosa.
In addition, we tracked nitrination of specific gastric mediators
in vivo, namely pepsinogen and evaluated the impact of this
post-translational modification on protein (pepsin) function.

Wistar rats (n=4, per group) were used to monitor gastric nitra-
tion in vivo. The rats were divided in two groups: a healthy
(un-treated) group and another with acute gastric inflamma-
tion (induced by diclofenac - 30 mg/Kg). Ulceration developed
for 4 hours, after which the animals were killed. Animals from
both groups were further fed (or not) with nitrite 1.38 mg/Kg.
All drugs were given by oral gavage. Protein tyrosine nitrina-
tion was evaluated by immunohistochemistry and immuno-
precipitation.

Nitrotyrosine (NT) labeling was detected in the deep mucosa
of untreated rats suggesting that nitrination is a physiological
event in the stomach. NT yields increased in the stomach of
rats with acute gastric ulceration (p < 0.01) and were further
enhanced when they were subsequently fed with nitrite (p < 0.01).
NT staining was located within the lamina propria and
blood vessels but also in cells of the oxyntic glands, where an
intense cytoplasmatic staining suggests nitrination of specific
gastric mediators stored in cytoplasmatic vesicles, such as
pepsinogen (fig. 1). Pepsinogen nitrination occurs under basal
conditions but increases under gastric ulceration. In this later
case, nitrite further enhanced nitrination yields (p < 0.05)(fig.2)
but nitrite-fed rats showed reduced levels of both, overall and
pepsinogen nitrination in respect to untreated rats. Pepsinogen
nitrination has implications to the proteolytic function of the

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derived pepsin as its activity is c.a. 5 times lower than the one derived from non nitrated pepsinogen (p < 0.001). We demonstrate that pepsinogen is nitrated under physiological conditions in the stomach and that nitration yields increase under acute ulceration. Moreover, nitrite increases the nitration yields under inflammatory events but, in the healthy stomach, it decreases the efficiency of nitration, thus having a dual role depending on the health and disease status. We also provide evidence that pepsinogen nitration impairs the proteolytic function of the derived pepsin, thereby impacting on protein function. Expectedly, an inefficient protease would impair the digestion of dietary proteins but, on the other hand, would also prevent the breakdown of endogenous proteins (mucins, collagen) vital for gastric integrity, thus preventing peptic ulcer disease.

Impact of dietary nitrite on pepsinogen nitration in healthy and ulcerated stomachs in vivo. (A) Pepsinogen is shown to be nitrated under physiological conditions (lane 4), but nitration yields increase under inflammatory states (lane 2). The exposure of stomachs with active gastric ulcers to physiological concentrations of nitrite further increases pepsinogen nitration (lane 1). Dietary nitrite decreases the levels of nitrated pepsinogen in healthy stomachs (lane 3). Pepsinogen was immunoprecipitated by an anti-pepsinogen polyclonal antibody and nitration was detected by western blot using an anti-nitrotyrosine antibody. The densitometric analysis of the data is depicted in figure (B). Values are means±SEM of 4 rats per condition (*p<0.05).

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C27

Lipopolysaccharide-induced endotoxaemia reduces the maximal rate of mitochondrial ATP production in rat skeletal muscle and this effect is specific to the pyruvate dehydrogenase complex

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We have provided evidence of a role for the inhibition of mitochondrial pyruvate dehydrogenase complex (PDC) in the impairment of muscle carbohydrate oxidation in vivo during lipopolysaccharide (LPS)-induced endotoxaemia in the rat(1)(2) and that pro-inflammatory cytokine mediated upregulation of PDK4 transcription is likely to play an important role in this response(3). The aim of the present study was to determine the impact of LPS administration on maximal rates of mitochondrial ATP production and components of the electron transport chain in rodent muscle.

Under general anaesthesia (fentanyl citrate/medetomidine 300μg.kg-1 each) Sprague-Dawley rats were implanted with a jugular vein catheter. Reversal/analgesia was provided with atipamezole 1mg.kg-1/buprenorphine 0.02mg.kg-1. Prepared rats received a continuous intravenous infusion of LPS (15 μg.kg-1.h-1) (n=8) or saline for 24 h at 0.4 ml.h-1 (n=7). Animals were terminally anaesthetised with thiobutabarbital sodium (80 mg.kg−1) and the soleus muscles removed. A bioluminescence technique was used to measure maximal rates of ATP production in isolated mitochondrial suspensions in the presence of a variety of substrates. The activity of components of the mitochondrial electron transport chain, PDC activation status and muscle ATP and lactate content were also determined. Values in the text and Figure 1 represent mean±SEM and statistical comparisons across treatment groups were performed using Student’s unpaired T-test.

Muscle ATP content was reduced (control 18.72 ± 1.21 vs LPS 12.96 ± 1.62 mmol.kg-1dm, p<0.05) and lactate content increased (control 2.44 ± 0.82 vs LPS 5.28 ± 0.74 mmol.kg-1dm, p<0.05) following LPS compared to control. Muscle PDC activation status was reduced following LPS compared with control (control 0.46 ± 0.05 vs LPS 0.27 ± 0.05 mmol.min-1.mg protein-1, p<0.05). Mitochondrial ATP production rate in LPS treated animals was reduced compared to control when pyruvate was used as a substrate, but the corresponding rates for the other substrates tested was no different between treatment groups (Figure 1). Assays of mitochondrial complexes (NADH-cytochrome c reductase, succinate dehydrogenase, succinate-cytochrome c reductase and cytochrome c oxidase) revealed no differences in activity between treatment groups.
In keeping with previous work(1)(2)(3) LPS administration reduced PDC activation status and increased muscle lactate accumulation. In accordance with this, the maximal rate of mitochondrial ATP production from pyruvate was reduced by 45%. Endotoxaemia therefore impairs skeletal muscle mitochondrial function, and this effect appears to be specific to PDC. Strategies to improve muscle carbohydrate oxidation and mitochondrial function during endotoxaemia should be targeted towards PDC.

We used the(Cck-EGFP)BJ203Gsat transgenic mouse model in which enhanced Green Fluorescence Protein(eGFP) is expressed under the control of the CCK gene promoter. Cryosections of duodenum were immunostained with an anti-mouse proCCK antibody. Duodenal epithelial cells were dissociated (from 4 adult mice per experiment), using a mechanical/chemical method and analyzed by fluorescence activated cell sorting (FACS). Living cells were sorted into two populations, one eGFP-positive (eGFP+) and one eGFP-negative (eGFP-). RNA was isolated from sorted cells and its integrity was analyzed on a PicoChip Bioanalyzer. After DNAse treatment, RNA was subjected to semi-quantitative RT-PCR.

Duodenal eGFP cells are flask-shaped with a narrow apical and a broad basolateral membrane, typical of entero-endocrine cells.90% of eGFP-tagged cells were co-stained with an anti-CCK antibody, confirming that eGFP cells express CCK. CCK staining was more intense at basolateral region, where secretory vesicles are localized. Dissociated duodenal cells were subjected to FACS. 15000-25000 eGFP+ cells (0.3-0.7 % of total population) were isolated in each experiment. Fluorescence microscopy confirmed that the purity of eGFP+ sorted cells was higher than 90%.Semi-quantitative RT-PCR revealed that CCK mRNA was exclusively expressed in eGFP+ cells. Moreover, RT-PCR of several transcripts that are specifically expressed in goblet and epithelial cells confirmed that we obtained a pure I-cells population with minimum contamination. Then, we examined if I-cells express long chain fatty acid(LCFA) receptors GPR40 and GPR120, finding that GPR40 and GPR120 mRNA transcripts are enriched in I-cells. We also investigated if the oleoylethanolamide receptor GPR119 is present in I cells and found that GPR119 is enriched in I cells. These experiments were repeated 3 times with similar results. We report that I-cells contain mRNA transcripts of GPR40,GPR119 and GPR120.Presence of GPR40 and GPR120 in I-cells indicates that they may act as fat sensors to promote CCK release. GPR119 presence in I-cells is surprising and may indicate that I cells are targets of endocannabinoid system.

We are indebted to Professor Graham Dockray (University of Liverpool) for anti-CCK antibody.

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### C28

**Murine I-cells express G-Protein Coupled Lipid Receptors GPR40, GPR119 and GPR120 mRNA transcripts**

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Cholecystokinin (CCK) is the archetypal anti-orexigenic and satiety hormone that co-ordinates digestion by inhibition of gastric emptying and release of pancreatic enzymes and bile. Following ingestion of fat, CCK is secreted by a subset of entero-endocrine cells (I-cells) that are localized in small intestine. Isolation of native I-cells will help us to understand how signals from nutrients are transmitted in the cells to stimulate CCK release. The aim of our study was to isolate native I-cells and investigate the expression of G protein coupled receptors (GPCRs) that may be crucial for nutrient sensing.

**Figure 1. Maximal ATP production rates in soleus muscle of saline (control) and LPS treated rats using a variety of substrate combinations. *P<0.05, compared with control.**


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### C29

**The effects of syzygium aromaticum derived oleanolic acid on glucose transport across rat-everted intestinal sacs in vitro**

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The hypoglycaemic effects of Syzygium aromaticum derived triterpene, oleanolic acid (OA) in streptozotocin (STZ)-induced diabetic rats are mediated in part via increased hepatic glyco- gen synthesis [1]. The wide range of available anti-diabetic suggests that a variety of mechanisms of action are involved in the blood glucose lowering effects. The present study was designed to investigate the effects of OA on glucose transport in vitro using the everted rat intestinal protocol which has been previously described byMahomoodally et al., 2005. [2] Everted intestinal sacs from rats filled with 1 ml of Krebs–Henseleit...
bicarbonate buffer (KHB) were mounted in an organ bath containing 50 ml of the same incubation medium. D-glucose (10 mM) was added to the medium just before the start of the appropriate experiments. In separate preparations graded concentrations of either OA (0.375-3.00 mM) or the standard drug, phlorizin (10-6 - 10-3 M) were incubated for 30 min in the mucosal bathing fluid containing glucose (10 mM) to investigate effects on glucose transport across the intestine gut wall. The external incubation medium will be continuously bubbled with gas mixture of 95% oxygen) and 5% carbon dioxide during the whole incubation period. The organ bath was surrounded by a water jacket maintained at 37-40 °C. The transport of D-glucose was evaluated by measuring the increase in glucose concentration inside the intestinal sacs after 30 min of incubation. The change in glycolgen concentration in the gut wall was interpreted as assessed glucose metabolized. Graph Pad Instat software (version 4) using one way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test was used. P values < 0.05 were considered significant. Lower concentration (0.375 and 0.750 mM) of OA significantly inhibited (p < 0.05) D-glucose transport across the rat everted intestinal sac in a dose-dependent manner whereas the inhibitory effects of higher concentrations of 0.750, 1.500 and 3.000 mM could not be statistically separated. Phlorizin, however, exhibited dose-dependent inhibition of glucose uptake across the everted intestinal sac. The accumulation of glycogen concentration in the gut wall increased significantly in the presence of OA. The fate of glucose retained within intestinal wall is difficult to quantify since glucose is rapidly metabolized by enterocytes. We hypothesize that OA inhibits the active transport of d-glucose suggesting that the triterpene can be a potential alternative drug therapy of postprandial hyperglycaemia via inhibition of glucose uptake across the small intestine.


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C30

In vivo assessment of cardiac metabolism and functional derangement in the spontaneously hypertensive rat heart using hyperpolarized magnetic resonance spectroscopy and CINE-MR imaging

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Spontaneously hypertensive rats (SHRs) are a model of hypertension and develop pressure-overload concentric hypertrophy after 15 weeks [1]. One of the cellular responses to hypertension is a reduction in β-oxidation. It is hypothesised that in response the SHR heart switches to a glycolytic phenotype. The aim of this work was to assess in vivo metabolism in the hypertensive rat heart by performing magnetic resonance (MR) spectroscopy, using hyperpolarized [1-13C] and [2-13C]pyruvate. Cardiac functional and left ventricular mass (LVM) were assessed using CINE-MR. SHRs (n=13) were compared to control Wistar rats (n=11). Student two-tailed t-test were used to compare groups. On two separate days cardiac metabolism was assessed following a bolus injection of either hyperpolarized [1-13C] or [2-13C]pyruvate [2]. 1ml of 80mM hyperpolarized pyruvate was injected over 10s via a tail vein catheter into an anaesthetised rat positioned in a 7T MR scanner (2% isoflurane in O2 and N2O (4:1), 2l/min). On a third day cardiac function and hypertrophy were assessed using CINE-MR imaging. SHRs had a significant 63% increase in LVM compared to control (p<0.001). Cardiac function was altered in the SHR heart, with an increase in stroke volume and diastolic dysfunction in the atrial filling phase. A significant 85% increase in pyruvate dehydrogenase (PDH) flux (assessed using the sum of the 13CO2 and [1-13C]bicarbonate resonances detected following injection of [1,13C]pyruvate) was detected in the SHR hearts compared with controls (p<0.01). No difference in 13C label incorporation into lactate and alanine was observed between groups. [2-13C]pyruvate was used to assess 13C label incorporation into downstream TCA cycle metabolites. Incorporation into acetyl carnitine (p<0.001), glutamate (p<0.01) and citrate (p<0.05) pools was significantly increased within SHR hearts. However when these data were normalized to account for the increased PDH flux, no change in normalized label incorporation was observed. This implies the increase in acetyl carnitine, glutamate and citrate resonances was derived from increased production of [2-13C]acetyl-CoA and represents normal flux through the TCA cycle. This study shows an increase in PDH flux in the in vivo hypertensive heart. There is normal flux of metabolites within the TCA cycle, indicating no deficiency in energy production in the SHR heart. Cardiac functional data shows hallmarks of adaption to hypertension and hypertrophy. This study has not shown a switch to a glycolytic phenotype, as label incorporation into lactate remained unchanged between groups. However it does suggest a switch to increased glucose oxidation through PDH and the TCA cycle.

Figure 1: 13C label incorporation using [1-13C]pyruvate - The hypertensive heart has a significant increase in label incorporation into CO2 + bicarbonate, whilst no change is seen into lactate and alanine. (Means and S.E.M, * p < 0.01)


This study was supported by the British Heart Foundation, Medical Research Council and GE Healthcare.

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Human genetic mutations causing increased permeability to monovalent cations across the red cell membrane

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The ‘hereditary stomatocytes’ comprise a group of heterogeneous human haemolytic anaemias, in all of which the red cell membrane ‘leaks’ the monovalent cations sodium and potassium, compromising the osmotic balance in the cell (Stewart GW, 2004). Often, different conditions within the group can usefully be distinguished on the basis of the temperature dependence of the increased cation permeability. So far we have identified three human genes that can be mutated in these conditions. These are: SLC4A1, coding for the band 3 anion exchanger, AE1 (Bruce LJ et al., 2005); RHAG, coding for the Rh-associated glycoprotein, a probable gas transporter (Bruce LJ et al., 2009); and SLC2A1, coding for the GLUT1 glucose transporter (JF Flatt, H Guizouarn, NM Burton, F Borgese, RJ Tomlinson, RJ Forsyth, SA Baldwin, BE Levinson, P Quittet, P Aguilar-Martinez, J Delaunay, GW Stewart, LJ Bruce, unpublished).

All of these proteins are integral membrane proteins with 10-12 membrane-spanning domains, which are expressed in the red cell membrane. In no case is the ‘physiological’ substrate a cation, but something quite different: an anion, a gas, or a carbohydrate. The mutations are either substitutions or deletions of single amino acids. In each case we have shown that the mutant protein, when expressed in oocytes, carries an excess of cations.

These are all high-copy-number proteins of the red cell membrane (>500,000 per cell). Red cells have very slow cation transport rates (there are only about 350 NaK pump units cell−1 per cell). The cation leak turnover number of these mutants must be much lower than the turnover for the physiological substrates (>500,000 per cell). Red cells have very slow cation transport rates (there are only about 350 NaK pump units cell−1), and the mutation ‘leaks’ the monovalent cations sodium and potassium, compromising the osmotic balance in the cell.

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C33

Hydroxylases regulate epithelial Modulator of Na⁺/K⁺-ATPase expression: implications for intestinal fluid and electrolyte transport function

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Cl secretion, the primary driving force for intestinal fluid secretions, can become dysregulated in conditions of disease leading to the onset of diarrhea. Our previous studies have shown that inhibition of hydroxylases, which are the primary intracellular sensors of O₂ availability, exerts anti-secretory effects in vitro and in vivo through inhibition of Na⁺/K⁺-ATPase activity. Modulator of Na⁺/K⁺-ATPase (MONaKA) has been recently identified as an endogenous protein that regulates Na⁺/K⁺-ATPase activity in the nervous system by binding to the β subunits of the pump. Aim: Here we sought to investigate a potential role for MONaKA in mediating hydroxylase-induced attenuation of Na⁺/K⁺-ATPase activity in the colonic epithelium. Methods: Dimethylallyl glycine (DMOG) was used to inhibit hydroxylases. Ion transport was measured as changes in short-circuit current across voltage-clamped T₈₄ cell monolayers. Protein expression and localisation was measured by Western blotting and confocal microscopic analysis. mRNA expression was measured by RT-PCR. Results: MONaKA is highly expressed in T₈₄ colonic epithelial cells both at the mRNA and protein levels. Confocal imaging revealed that the protein is localised to the basolateral compartment of T₈₄ cells and colonic crypts isolated from mice, rats, and humans. Treatment of T₈₄ cells with DMOG (1 mM; 24hrs) increased expression of MONaKA to 190 ± 18.5% of that in control cells (n = 20, ***p < 0.001) 24 hrs after treatment. DMOG also increased protein expression of the regulatory β₁ subunit of the Na⁺/K⁺-ATPase to 173.8 ± 17.3 % of controls with a time course coincident to that of its effects on MONaKA (n = 3, *p < 0.05). MONaKA co-immunoprecipitated with both the catalytic α₁ and regulatory β₁α₂ subunits of the Na⁺/K⁺-ATPase. Finally, pre-treatment of cells with either actinomycin D (250ng/ml) or cycloheximide (5μM) did not alter DMOG-induced increases in MONaKA expression nor did they reverse the anti-secretory effects of DMOG in Ussing chambers. Conclusion: Hydroxylases regulate basolateral MONaKA expression in colonic epithelial cells by a posttranslational mechanism. In turn, increased association of MONaKA with the regulatory β₁ subunit attenuates Na⁺/K⁺-ATPase pump activity, thereby inhibiting epithelial secretory function. Our data have important implications for our understanding of how epithelial fluid and electrolyte transport can be regulated under physiological and pathophysiological conditions and suggest that hydroxylases may be good targets for the development of new drugs to treat intestinal transport disorders.

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C34

Identification of two cleavage sites in the γ-subunit of the epithelial sodium channel (ENaC) with functional importance for its proteolytic activation by plasmin

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Proteolytic processing of ENaC is essential for channel activation [1]. Recently, we reported that plasmin can proteolytically activate ENaC which may contribute to renal sodium retention in nephrotic syndrome [2]. In mouse γENaC a putative plasmin cleavage site (K194) has been reported [3]. For rat ENaC an indirect stimulatory effect of plasmin via prostasin has been proposed [4]. The aim of this study was to identify cleavage sites in human γENaC that are functionally important for channel activation by plasmin. Sequence comparison of human and mouse ENaC suggested a putative plasmin cleavage site in human γENaC (K189). To study its functional relevance we generated a γK189A mutant by site-directed mutagenesis and expressed wild-type (wt) and γK189A-ENaC in Xenopus laevis oocytes. The γK189A mutation reduced but did not abolish the stimulatory effect of plasmin (10 μg/ml) on ENaC. In contrast, mutating a putative prostanin site (γRRK178AAAA) had no apparent effect on the stimulatory response to plasmin. Interestingly, a double mutation (γRRK178AAAA; K189A) abolished the stimulatory effect of plasmin and also significantly reduced the stimulatory effect of chymotrypsin (2 μg/ml). Using a biotinylation approach, we investigated the appearance of ENaC cleavage products at the cell surface of wt or double mutant ENaC expressing oocytes treated with chymotrypsin for 5, 30, or 60 min. The time-dependent appearance of cleavage products correlated well with the stimulatory effect of chymotrypsin on ENaC currents and was significantly delayed in oocytes expressing the double mutant ENaC. Delayed proteolytic activation of the mutant channel was confirmed in single-channel recordings from outside-out patches. These experiments also demonstrated that the double mutation did not affect the single-channel conductance of ENaC. Moreover, they demonstrated that the activation of both wt and mutant channel by chymotrypsin was caused by a stepwise recruitment of so-called near silent channels.

In summary, the reduced stimulatory effect of plasmin on the wt or double mutant ENaC expressing oocytes treated with chymotrypsin may be good targets for the development of new drugs to treat intestinal transport disorders.
17β-Estradiol increases amiloride-sensitive Na\(^+\) current and PKCδ activity in renal collecting duct M1 cells

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17β-estradiol (E2), the most abundant oestrogen occurring naturally in the female body, regulates chloride secretion in distal colon in a gender-specific manner via inhibition of K\(^+\) recycling through basolateral K\(^+\) channels (1). The anti-secretory effect of E2 could provide an explanation for the fluid retention observed in females during periods of high circulating plasma oestrogen. The effect of E2 on ion transport in other organs contributing to electrolyte and fluid homeostasis in females has not been characterised. For example, oestrogen modulation of Na\(^+\) re-absorption in the renal cortical collecting duct could contribute to fluid conservation observed during periods of high circulating plasma oestrogen. The aim of this study was to determine whether E2 had an effect on Na\(^+\) re-absorption in the renal cortical collecting duct using the well-characterised M1-CCD cell line and to reveal the molecular mechanisms underlying effects of E2 on the amiloride-sensitive epithelial Na\(^+\) channel ENaC. We found that treatment with E2 (25nM) for 15 min increased the amplitude of the amiloride-sensitive current in M1-CCD cells grown as polarized monolayers in Ussing chambers (Control 1.5±0.4 μA/cm\(^2\), E2 2.8±0.4 μA/cm\(^2\); n=6). Experiments in amphotericin-B perforated epithelia using ouabain demonstrated that this response was not attributable to changes in Na\(^+\)/K\(^+\) ATPase pump current (Control 2.9±0.2 μA/cm\(^2\), E2 3.3±0.8 μA/cm\(^2\); n=4). Previous work from our laboratory has shown that the anti-secretory effect of E2 in distal colon is PKC-δ dependent (1). To test the role of PKCδ in the E2 activation of ENaC we measured the phosphorylation state of the PKCδ autophosphorylation residue Ser643 following E2 treatment. E2 stimulated PKCδ activity in a concentration-dependent manner and within a similar time frame as the E2 stimulatory effect on the amiloride-sensitive current. We found that PKCδ autophosphorylation was also stimulated by the ERα selective agonist PPT (1nM) but not by the ERβ agonist DPN (5nM). The stimulatory effect of E2 on PKCδ autophosphorylation was blocked by pre-treatment with the matrix metalloproteinase (MMP) inhibitor GM6001 (1μM) and by the EGFR inhibitor AG1478 (10μM). Moreover, E2 was unable to increase the amplitude of the amiloride-sensitive current when M1-CCD cells were pre-treated with the PKCδ inhibitor rottlerin (5μM) (E2 2.4±0.4 μA/cm\(^2\), E2 + rottlerin 0.6±0.3 μA/cm\(^2\); n=6). In conclusion, E2 treatment rapidly and concurrently increased the amplitude of the ENaC current and stimulated PKCδ activity in M1-CCD cells. These rapid responses to oestrogen were transduced via ERα and required the MMP-mediated transactivation of EGFR. O’Mahony F et al. (2007). J Biol Chem 282(34), 24563-24573. Funded by the Higher Education Authority of Ireland (PRTLI Cycle 4) through the National Biophotonics and Imaging Platform Ireland.

Urinary aminopeptidase activities as early and predictive biomarkers of renal dysfunction in cisplatin-treated rats

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Introduction: There is an urgent need for better biomarkers to permit more timely diagnosis of acute kidney injury (AKI), prediction of injury severity, and safety assessment during drug development (1). This study analyzes the fluorimetric determination of alanyl- (Ala), glutamyl- (Glu), leucyl-cystinyl- (Cys) and aspartyl-aminopeptidase (AspAp) urinary enzymatic activities as early and predictive biomarkers of renal dysfunction in cisplatin-treated rats. Methods: Male Wistar rats (n=5 each group) received a single subcutaneous injection of either saline or cisplatin 3.5 or 7 mg/kg, and urine samples were taken at 0, 2, 3, 7, 10 and 14 days after treatment. In urine samples we determined Ala, Glu, Cys and AspAp activities, proteinuria, N-acetyl-β-D-glucosaminidase (NAG), albumin, citatin and β2-microglobulin. Plasma creatinine and creatinine clearance were measured at the end of the experiment. All experimental procedures were performed according to the European Union Guidelines to the Care and Use of Laboratory Animals and approved by the Ethical Committee of the University of Jaén. Blood samples were obtained from left ventricle under terminal anesthesia (pentobarbital, 50 mg/kg, i.p.) and then killed with an overdose of pentobarbital (150 mg/kg, i.p.). Kidneys were removed and fixed in formalin solution for morphological analysis. Results: AlaAp, albumin and citatin were increased at the second day of treatment in the cisplatin 3.5 mg/kg treated group, showing their ability to detect a slight renal damage. All aminopeptidase activities and urinary biomarkers were significantly increased at the third day of treatment in the cisplatin 7 mg/kg treated group. At this point, AlaAp (r2=0.988; p=0.0001), CysAp (r2=0.992; p=0.0003), GluAp (r2=0.951; p=0.0046), NAG (r2=0.863; p=0.0225) and total protein (r2=0.787; p=0.0446) urinary excretion correlated with plasma creatinine level at the end of the experiment and could be considered as predictive biomarkers of renal injury severity. Cisplatin significantly enlarged the fibrosis area in the tubular interstitium of the kidney, as observed by other authors (2) and it was dose-dependent. ROC Area under the curve (AUC) for aminopeptidase activities at day 3 was >0.5. Conclusion: Ala, Cys, Glu and AspAp enzymatic activities are urinary biomarkers of the AKI induced by cisplatin. AlaAp activity is an early biomarker and Ala, Cys and GluAp activities are also predictive biomarkers of renal dysfunction. These determinations can be very useful in the prognostic and diagnostic of renal dysfunction in preclinical research and clinical practice.


This study was supported by a grant (R1/12/2010/66) from the University of Jaén and from the Carlos III Health Institute of the Spanish Ministry of Health and Consumer Affairs (Red de Investigación Renal, REDinREN RD06/0016/0017 and RD07/0016/2008). “FEDER una manera de hacer Europa”.

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C37

The Pore Structure and Gating Mechanism of K2P Channels

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Two-pore domain (K2P) potassium channels are important regulators of cellular electrical excitability. However, the structure of these channels and their gating mechanism, in particular the role of the bundle-crossing gate, are not well understood. Here we report that quaternary ammonium (QA) ions bind with high-affinity deep within the pore of TREK-1 and have free access to their binding site prior to channel activation by intracellular pHi or pressure. This demonstrates that, unlike most other K+ channels, the bundle-crossing gate in this K2P channel is constitutively open. Furthermore, we used QA ions to probe the pore structure of TREK-1 by systematic scanning mutagenesis and comparison of these results to different possible structural models. This revealed that the TREK-1 pore most closely resembles the open-state structure of KvAP. We also found that mutations close to the selectivity filter and the nature of the permeant ion profoundly influence TREK-1 channel gating. These results demonstrate that the primary activation mechanisms in TREK-1 reside close to, or within the selectivity filter and do not involve gating at the cytoplasmic bundle-crossing.

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C38

Right ventricular origin of arrhythmias in Brugada Syndrome is due to lower Na+ channel expression and function

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Brugada syndrome (BrS) is associated with loss of Na+ channel function and ventricular tachycardia originating in the right ventricle (RV). Two major mechanisms have been postulated: conduction delays (1) or increased repolarization heterogeneities (2). We use a heterozygotic Scn5a+/− murine model, which has previously shown both conduction and repolarization abnormalities consistent with BrS (3, 4), to assess the underlying molecular mechanisms.

Expression levels of mRNA for Na1.5 and a range of K+ channels were measured from the LV and RV of homogenised WT and Scn5a+/− hearts using real time RT-PCR and equivalent protein levels measured by Western blotting. Action potential durations (APDs), fast Na+ currents (IfNa), persistent Na+ currents (IfpNa) and transient outward K+ currents (Ito) were measured in isolated myocytes.

Na1.5 mRNA and protein expression were both lower in Scn5a+/− than WT, but particularly so in the RV (protein expression relative to WT LV: 0.91±0.05 vs 0.53±0.07, p<0.05, n=4). While there were no significant differences in mRNA or protein expression for Kir2.1, K1.4 or K1.5, there were significantly lower mRNA and protein levels of K1.4 in RV than LV in both WT and Scn5a+/−, and higher protein levels also for K4.3 and KChIP2 (protein expression in Scn5a+/− relative to WT LV: 1.90±0.09 vs 1.04±0.08 (K4.2), 1.75±0.06 vs 1.10±0.06 (K4.3), 1.20±0.08 vs 0.77±0.09 (KChIP2), p<0.05, n=4).

APDs were smaller in the RV than LV of both WT and Scn5a+/−, but with a larger intraventricular gradient in the Scn5a+/− murine model, and upstroke velocity was decreased in the RV of Scn5a+/− isolated hearts. IfNa currents were greater in the RV of both WT and Scn5a+/− hearts (WT: 22.5±2.7 pA/pF vs 32.4±0.3 pA/pF, p<0.05, n=16), with similar I-V relationships, kinetics and voltage dependence of activation and inactivation. Max Ito, was similar in LV (−44.0±1.6 pA/pF) and RV (−44.7±0.7 pA/pF) of WT hearts, but was significantly decreased to −31.8±1.3 pA/pF in the LV and to −20.9±1.0 pA/pF in the RV of Scn5a+/− (p<0.05, n=12). The I-V relationship and voltage dependence of activation were unchanged, but inactivation was shifted to more positive values (RV: V1/2 of −91.0±0.7 mV vs −74.2±0.3 mV, p<0.05, n=12). Max ItpNa was also decreased in a similar pattern in Scn5a+/− hearts.

Our findings suggest that in the LV there is upregulation of the single Scn5a allele, which does not occur in the RV. The reduced expression of Na+ currents in the RV leads to smaller IfNa currents, which results in slowed conduction, and smaller ItpNa currents, which in combination with increased Ito currents, results in shorter APDs and greater heterogeneity of repolarization. These insights could prove useful in finding new drug treatments for a disease where the current mainstay of therapy is cardioverter defibrillator implantation.
Oral Communications

Figure 1. Na\textsubscript{v}1.5 protein expression in LV and RV of WT and Scn5a\textsuperscript{+/-} hearts. (A) Representative Western blot. (B) Graph comparing mean relative expression levels normalised to myosin expression. Significant differences: * effect of genotype; # effect of ventricle.

Figure 2. I\textsubscript{Na} current measurements. (A) Representative traces in myocytes from LV and RV of WT and Scn5a\textsuperscript{+/-} hearts. (B) I-V relationship. (C) Max I\textsubscript{Na} densities. Significant differences: * effect of genotype; # effect of ventricle.

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Blebbistatin - the excitation-contraction uncoupler significantly affects cardiac electrophysiological and calcium homeostasis

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Purpose: Movement artefacts pose challenge in optical mapping studies. Blebbistatin (BS) is a recently discovered inhibitor of myosin II isoform and has been adopted as the mechanical uncoupler of choice for optical mapping. Previous studies in cellular and whole heart preparations suggested that BS has no direct electrophysiological effects. Our aims were to 1) measure the effects of BS on monophasic action potential duration (MAPD), maximum slope of MAPD restitution (RT), ventricular effective refractory period (ERP), ventricular fibrillation threshold (VFT), Ca\textsuperscript{2+} transient duration (CaTD) and optically recorded action potentials and 2) to determine if BS would alter the effect of sympathetic nerve stimulation (SNS) on ERP and VFT.

Methods:
Aim 1: Langendorff perfused hearts (NZW rabbits, n=8) were obtained after pentobarbitone euthanasia (160mg/kg, i.v.). Left ventricular (LV) MAPD (90% decay) was measured during constant ventricular pacing (300ms CL). RT was obtained using a single extra-stimulus protocol with maximum slope measured using contact electrodes. VFT was measured as the minimum current required inducing sustained VF with burst pacing (30x30ms). LV epicardial Ca\textsuperscript{2+} transients were measured using Fura-2 AM fluorescence and CaTD measured at 90% decay. Optical action potentials (APs) were recorded using 5\muM Di-4-ANEPPS.

Aim 2: Measurements were taken in the absence and presence of SNS (4.8Hz, 4.9V, n=3) in the isolated innervated rabbit heart. Following pre-sedation (ketamine (10mg/kg), medetomidine hydrochloride (0.2mg/kg) and butorphanol (0.05mg/kg) i.m.), general anaesthesia was established with propofol (1%w/v ad libitum, i.v.) during which vessels were ligated and cervico-thoracic tissues isolated to give the innervated heart preparation (1). Animals were euthanised with pentobarbitone overdose (160mg/kg, i.v.). The resulting ex vivo preparation was perfused via the descending aorta with constant flow.

All measurements were taken at baseline (BL) and after 60min perfusion of BS-5\muM. Data are mean±SEM, *P<0.05 using t-test.

Results:
Aim 1: BS significantly prolonged MAPD (113.2±2.2 to 163.2±3.1ms, [Fig 1A]) and ERP (135.0±4.1 to 166.7±2.5ms) whilst increasing RT slope (0.60±0.08 to 1.45±0.27) and VFT (3.5±0.5 to 12.9±3.8mA). CaTD (Fig 1B) and optical APs (Fig 1C) were similarly prolonged.

Aim 2: Although there was a trend for BS to alter the percentage change in SNS induced shortening of ERP or decrease in VFT (Figure 2A-B), this failed to reach significance. Conclusion: BS had significant effects on ventricular electrophysiology and Ca\textsuperscript{2+} homeostasis in the isolated heart. BS caused a non-significant reduction on the ventricular electrophysiological response to SNS. The use of BS should be treated with caution in optical mapping studies.
Influence of connexin expression and co-expression levels on action potential propagation in the HL-1 cells

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In the myocardium gap junctions mediate the orderly spread of current flow from cell-to-cell on which the sequential contraction of the cardiac chambers depends on. In mammalian hearts gap junction proteins connexin43 (Cx43), Cx40 and Cx45 are co-expressed in distinctive combinations and relative quantities in functionally specialised subsets of cardiac myocytes. Numerous studies in heart failure have identified a loss of connexin expression as key contributors to arrhythmias alongside factors such as membrane excitability and tissue architecture. The functional consequences of connexin co-expression in modulating action potential propagation in vivo are poorly understood. To study the relative importance of voltage-gated ion channels and gap junctions in relation to propagation velocities, clones of the original HL-1 mouse atrial myocyte tumour line were used as an in vitro cell model. The values presented are means ± standard error of the mean, compared by an unpaired t test or ANOVA. Five clones were characterised for expression of myocytic markers, calcium handling proteins and connexins. Using microelectrode arrays, two of the clones, #2 and #6, displayed large differences in conduction velocities (#2: 4±1mm/s\(^{-1}\), n=6 #6: 41±2mm/s\(^{-1}\), n=13; p<0.001). Patch clamp recordings showed similar membrane current densities in both clones for sodium (#2: -77.5±6.0pA/pF; #6: -85.0±14.3pA/pF; p>0.05), L-type (#2: -0.89±0.07pA/pF; #6: -0.86±0.04pA/pF; p>0.05) and T-type (#2: -0.94±0.04pA/pF; #6: -0.89±0.03pA/pF, p>0.05) calcium channels. However, large differences were seen in the expression levels of Cx43, Cx40 and Cx45 between the two clones. RNA interference combined with microelectrode arrays was employed in clone 6 to establish the relative importance of each connexin in impulse propagation. Knockdown of Cx40, Cx43 or Cx45 resulted in a decrease in conduction velocity but the loss in conduction was connexin dependent (37±2mm/s\(^{-1}\) in control n=6; 28±2mm/s\(^{-1}\) in Cx43 knockdown, n=8; 24±2mm/s\(^{-1}\) in Cx40 knockdown n=10; 16±2mm/s\(^{-1}\) in Cx45 knockdown, n=3; p<0.05). Simultaneous knock down of two or more connexins caused a similar and not additive decrease in conduction velocities (21±2mm/s\(^{-1}\) in Cx43+Cx40 knockdown, n=3; 23±1mm/s\(^{-1}\) in Cx43+Cx45 knockdown, n=3; 22±1mm/s\(^{-1}\) in Cx40+Cx45 knockdown, n=3 and 23±2mm/s\(^{-1}\) in Cx43+Cx40+Cx45 knockdown, n=3; p<0.05). These results indicate that electrical coupling by gap junctions is the major determinant of conduction velocities, particularly Cx45 in the HL-1 clones. Further experiments on clone 6 are currently ongoing by dual cell patch clamp to assess the total gap-junctional conductance before and after RNA interference.

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Nav1.5 deficiency triggered TGF-β1-mediated fibrosis as a key mechanism producing sinus node dysfunction associated with Scn5a disruption and aging in mice

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Mutations in the cardiac Na+ channel gene (SCN5A) can adversely affect electrical function in the heart but effects can be age-dependent (Lei et al. 2007). We explored for interacting effects of Scn5a-disruption and aging on the pathogenesis of sinus node dysfunction (SND) in a heterozygous Scn5a knockout (Scn5a+/−) mouse model. We compared in vivo and ex vivo electrical functional, histological and molecular features in young (3-4 month) and old (1 year) wild type and Scn5a+/− mice. In vivo senatorial node (SAN) electrophysiological studies in anaesthetized animals with intraperitoneal injection of 2,2,2-Tribromoethanol (250 mg/kg). ECG was recorded using subcutaneous electrodes with connection to Powerlab 26T system using the Chart v6.0 program (AD Instruments).

Both Scn5a-disruption and aging were associated with decreased heart rate variability, reduced sinoatrial node (SAN) automaticity and slowed sinoatrial conduction. Thus, both aging and Scn5a-disruption significantly prolonged both intrinsic cycle length (CL) (CL in ms: young WT, 164±8; n=5; young Scn5a+/-, 211±12, n=7; old WT, 231±25, n=8; old Scn5a+/-, 321±30, n=7) and sinoatrial conduction time (SACT) (SACT in ms: young WT, 8.0±0.5, n=5; young Scn5a+/-, 11.4±0.7, n=7; old WT, 12.0±1.1, n=8; old Scn5a+/-, 21.9±1.9, n=7). Furthermore, these effects interacted to produce the greatest functional changes in the old Scn5a+/- hearts (CL: Scn5a+/- vs. WT, P<0.005; old vs. young, P<0.001. SACT: Scn5a+/- vs. WT, P<0.001; old vs. young, P=0.001. Interaction of the two effects, P = 0.04). They also led to increased collagen and fibroblast levels and up-regulated TGF-β1 and vimentin transcripts providing measures of fibrosis, and reduced Nav1.5 expression. All these effects were most noticeable in old Scn5a+/- mice. Na+ channel inhibition by E3-Nav1.5 antibody directly produced measures of fibrosis, and reduced Nav1.5 expression effects, P=0.04). They also led to increased collagen and fibroblast levels and up-regulated TGF-β1 and vimentin transcripts providing measures of fibrosis, and reduced Nav1.5 expression. All these effects were most noticeable in old Scn5a+/- mice. Na+ channel inhibition by E3-Nav1.5 antibody directly increased TGF-β1 production in both cultured human cardiac myocytes and fibroblasts. Finally, aging was associated with down-regulation of a wide range of ion channel and related transcripts, again greatest in old Scn5a+/- mice. The quantitative results from these studies permitted computer simulations that successfully replicated the observed SAN phenotypes shown by the different experimental groups. These results implicate a tissue degeneration, triggered by Nav1.5-deficiency, manifesting as a TGF-β1 mediated fibrosis accompanied by electrical remodelling, in the SND associated with Scn5a-disruption or aging. The latter effects interact to produce the most severe phenotype in old Scn5a+/- mice. In demonstrating this, our findings suggest a novel regulatory role for Nav1.5 in cellular biological processes additional to its electrical function.


The work was supported by the Wellcome Trust (project grants: 081283, 081809), British Heart Foundation (PG/08/006/24399), A Cardiovascular Therapeutics Inc. (USA) research grant and the Manchester Biomedical Research Centre, University of Manchester.

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Influence of ovarian cycle on muscle metaboreflex control of spontaneous cardiac baroreflex sensitivity in young women


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The partial restriction of blood flow to the dynamically exercising skeletal muscles decreases spontaneous cardiac baroreflex sensitivity (cBRS) in dogs. The augmented activation of metabolically-sensitive skeletal muscle afferents (i.e. muscle metaboreflex) might explain this apparent reduction in cBRS, however whether this occurs in humans remains unclear. Accumulating evidence suggests that ovarian hormones may modulate resting baroreflex sensitivity and the muscle metaboreflex mediated cardiovascular responses to exercise. However, it is presently unknown whether ovarian cycle influences muscle metaboreflex control of spontaneous cBRS.

To examine this, 11 healthy women not taking oral contraceptives (age 20±1 years, weight 61±3 kg, height 164±2 cm) were studied during two phases of their ovarian cycle: the early follicular phase (days 1-5) and the late follicular phase (days 10-15; identified using an ovulation kit). Steady-state leg cycling exercise was performed at low (90 bpm; 19±3 Watts) and moderate (120 bpm; 70±5 Watts) intensities, under free-flow conditions and with muscle metaboreflex activation induced by bilateral thigh cuff inflation (100 mmHg). Heart rate (HR; ECG) and beat-to-beat blood pressure (BP, Portapress) were continuously monitored. Spontaneous cBRS was estimated using the sequence technique. Statistical analyses were performed using two-way repeated-measures ANOVA with Bonferroni post hoc tests.

Leg cycling under free-flow conditions induced an intensity dependent increase in HR (P<0.05), while BP remained unchanged from rest. Muscle metaboreflex activation markedly increased HR and BP at low (+12±2 bpm and +14±2 mmHg) and moderate (+19±2 bpm and +19±2 mmHg) exercise workloads (P<0.05). Leg cycling under free-flow conditions tended to evoke an intensity dependent reduction in cBRS (21±5, 7±1 and 1±0.1 ms/mmHg at rest, low and moderate workloads, respectively). Muscle metaboreflex activation reduced cBRS during low intensity exercise (-2.0±0.5 ms/mmHg, P<0.05), but not during moderate intensity exercise (-0.1±0.1 ms/mmHg, P>0.05). The HR, BP and cBRS responses to leg cycling exercise under free-low conditions, or with muscle metaboreflex activation, were no different during early and late follicular phases. Our data suggest that partial restriction of blood flow to the dynamically exercising skeletal muscles (i.e. muscle metaboreflex activation) decreases spontaneous cBRS during low intensity exercise in young women. However, muscle metaboreflex control of spontaneous cBRS is similar during the early and late follicular phases of the ovarian cycle.
The effect of ethyl alcohol on contractions of isolated mammalian skeletal muscle fibres

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Muscle strength is reversibly decreased with acute and chronic alcohol ingestion (Urbano-Marquez et al. 1989). In vitro ethanol has been shown to decrease both isometric twitch and tetanic contractions in isolated rat diaphragm preparations and isolated frog muscle fibres (Khan 1981, Pagala et al. 1995). Current evidence suggests that this alcohol induced depression of force is via effects on cell membrane properties or by effects on Ca²⁺ channels in the sarcoplasmic reticulum. It is not known if there are any direct effects of alcohol on the actomyosin contractile process. Here we examine the effect of alcohol on contractions of isolated intact and skinned mammalian muscle fibres.

Adult male Wistar rats (275-350g) were killed with an intraperitoneal injection of an overdose of Sodium Pentobarbitone (150mg kg⁻¹). Intact fibre bundle preparations from rat flexor hallucis brevis muscle were prepared and mounted in a trough system between a motor and a force transducer (Coupland & Ranatunga 2003). Fibre bundles were bathed in physiological Ringer solutions with different ethanol concentrations (range 0.6% to 3%v/v). Twitch and tetanic tensions were recorded at 20°C. Chemically skinned fibres (using the detergent 0.5% Brij 58) were prepared from rabbit psoas muscle (killed by an intravenous overdose of Sodium Pentobarbitone (150mg Kg⁻¹)). Fibres were maximally Ca²⁺ activated by exposing them from a relaxing, to a pre-activating and then into an activating solution at 20°C (see Coupland et al. 2001) with different ethanol concentrations (range 0.5% to 3%v/v). Force transducer output was recorded using CED 1401 laboratory interface and Signal Averager software (Cambridge Design Ltd). Tension data put was recorded using CED 1401 laboratory interface and Sig.

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C45

CaMKII overexpression reverses the effects of electroproportion-induced damage on skeletal muscle twitch parameters

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Calcium/calmodulin-dependent protein kinase II is expressed in human skeletal muscle and activated during exercise (1). However, its function in skeletal muscle is still unclear. It has been hypothesised to be involved in regulating muscle phenotype and calcium release from, and uptake into, the sarcoplasmic reticulum, which would likely have functional consequences. We therefore investigated the effects of in vivo CaMKII overexpression on muscle force parameters of both fast-twitch and slow-twitch muscle. Female 3 month-old Wistar rats (190-225g, n=8) were anesthetised with 2-4% isoflurane through inhalation. Plasmids encoding α/β-CaMKII and a reporter plasmid were injected I.M. into the right m. gastrocnemius medialis (GM) and m. soleus (SOL) (CaMKII), while contra-lateral muscles were injected with the reporter only (Control). Plasmid injections were followed by electroporation and rats were kept in cages for 7 days afterwards. Muscle force parameters were measured in situ while rats were anaesthetised with 12.5% urethane (1.2ml/100g, injected I.P.) and rested on a heated pad. GM and SOL were isolated from surrounding muscles and their tendons connected to force transducers. The sciatic nerve was stimulated at supra-maximal intensity using a protocol consisting of twitches and a tetanic contraction (100Hz). Force data were recorded at 1000Hz. Time to peak twitch force (TTP), twitch half-relaxation time (HRT), maximum twitch force (Ftw) and maximum tetanic force (Fmax) was calculated from data obtained at optimum muscle length. The same contraction parameters were determined for a group of non-transfected muscles (NT; n=8). Data (absolute means ± S.E.M. are shown) were normalised to mean NT values. Effects of CaMKII overexpression (Control vs. CaMKII) and the electroporation procedure itself (NT vs. Control) were analysed with separate factorial ANOVA’s followed by paired and independent t-tests, respectively, for each muscle. Only intra-animal muscle pairs were used to determine the effects of CaMKII overexpression. Overall, electroporation increased TTP (p<.01), HRT (p<.01) and decreased Fmax (p<.01). This was associated with the presence of centrally nucleated fibres in the electroporated region. CaMKII overexpression decreased TTP (p<.01) and HRT (p<.02), but did not increase Fmax, significantly (p<.26). Ftw was not significantly affected by electroporation or CaMKII overexpression. Changes (Control vs. CaMKII) in SOL and GM followed similar trends (SOL: TTP

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C46


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this is consistent with joint torque acting against the gravity vector, possibly to increase joint stiffness and improve perceived stability. While small dynamic adjustments of configuration may have occurred continually, the broad multi-joint configuration remained stable and distinct from the onset of standing. This validates our previous findings that a postural set-point typical of standing exists, is aimed for and is defended (2,3).


Di Giulio I, Maganaris CN, Baltzopoulos V, & Loram ID. Human standing: does the postural set pre-program a rigid knee? Proceedings of The Physiological Society. 126, PCT2 (Manchester, 2010).

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C47

Is the standing configuration a postural set that is aimed for?

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Posture might be controlled via pre-programmed set points and feedback mechanisms associated with maintaining or changing body configuration (1). The processes involved in, and the adjustments associated with, gaining the standing configuration are revealed using perturbations. Lying down on a horizontal board was used to deprive the body of the vertical reference point to investigate the way the upright configuration goal was aimed for. The question asked was whether, after the individual was passively brought upright, the nervous system either (a) maintained the body configuration imposed by the board, or (b) sagged randomly into a position or (c) actively aimed for a different multi-joint point of equilibrium.

14 healthy participants, aged 47±13 years, laid horizontally on a table with feet against a perpendicular support for 5min and were then brought passively to the upright position by rotating the table until the feet on the support were planted onto a force plate. With the participant self supporting and posturally quiet, the vertical table was released from the foot support surface and wheeled away leaving the participant free-standing for 160s. A 10-camera motion analysis system measured body kinematics. The quantities calculated were: sagittal CoG; joint centres, flexion-extension and internal-external rotation angles of the ankle, hip and knee. Quantities were tested for difference between the time when the board was removed and “steady state” standing (160s later) (Kruskal-Wallis).

At the initiation of standing (i.e. board removed) hip and knee joint centres were in front of the CoG, the ankle was behind. During the 160s after the board was removed, all the quantities approached their final values asymptotically. We conclude that CaMKII overexpression mitigated or reversed the effects of electroporation-related damage on twitch characteristics. It remains to be determined whether this is due to acute effects or the result of structural adaptations.


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C48

Dual processing changes reactive balance in healthy adults

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It is widely accepted that attentional demands for postural control placed upon an individual vary according to the nature of the task, the age of the individual and their postural stability [1]. Dual tasking paradigms which combine postural and cognitive tasks can assess attentional demands for posture control, as well as the interference between these tasks [2, 3]. Currently, dual tasking is investigated by providing a single cognitive task alongside a postural activity (e.g. standing, walking or stepping over an obstacle) [4, 5]. How more responsive balance activities are affected by dual tasking remains unclear. This pilot study extends dual tasking to a dual processing paradigm. A reactive balance task is combined with responsive audio tasks.

Healthy adults (n=10, aged 26-61 years [mean 38.1]) with no evidence of balance impairment, hearing loss or colour blindness participated in this study. An instrumented floor with four colour coded directions (front, back, left and right) was developed to measure reactive balance. This stepping task required the subject to perform a directed step at the presentation of a colour stimulus (Fig 1). The secondary verbal or spatial cognitive task alongside a postural activity (e.g. standing, walking or stepping over an obstacle) [4, 5]. How more responsive balance activities are affected by dual tasking remains unclear. This pilot study extends dual tasking to a dual processing paradigm. A reactive balance task is combined with responsive audio tasks.

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Dual processing significantly increased mean step response time (F=22.511, p<0.01). Post-hoc analysis showed that the
spatial task significantly increased mean step response time by 67% compared to the stepping task on its own (p<0.01). However, the verbal task did not significantly increase mean step response time (p=0.14). Dual processing significantly increased mean response time for both verbal (p<0.05) and spatial (p<0.01) secondary tasks, with spatial task response times being significantly longer than verbal response times under dual processing conditions (p<0.01).

This study investigated the effect of added attentional demands by utilising a novel dual processing protocol on the timing and accuracy of stepping responses in healthy adults. Dual processing significantly increases step response times and response times for the secondary cognitive task; with concurrent processing of two spatial orientated tasks having the greatest effect.

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**Fig 1: Study Set up**


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**Oral Communications**

**C49**

**Does gastrin play a role in haematopoiesis?**

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**Introduction:** Gastrins are peptide hormones important for gastric acid secretion and growth of the gastrointestinal mucosa. We have previously demonstrated that ferric ions bind to gastrins, that the gastrin-ferric ion complex interacts with the iron transport protein transferrin in vitro, and that circulating gastrin concentrations positively correlate with transferrin saturation in vivo. These observations formed the basis for our proposal that circulating gastrins may act as chaperones for the uptake of ferric ions by apo-transferrin (1). Aim: To investigate the effects of long-term dietary iron deficiency in mice with altered circulating gastrin concentrations.

**Methods:** Gastrin-deficient (Gas/-) and hypergastrinemic cholecystokinin receptor 2-deficient (Cck2r/-) mice, both of which have reduced basal gastric acid secretion, were fed diets containing normal, low or high iron (160, 3 or 6000 mg of iron per kg wet weight, respectively). After 6 weeks, mice were anaesthetised by i.p. injection with ketamine (100 mg/kg)/xylazine (10 mg/kg), blood was collected by cardiac puncture, and iron status was assessed by measurement of serum ferritin and hepatic iron concentrations. Circulating concentrations of erythropoietin and thrombopoietin were measured by ELISA assay. Expression of hepatic Hamp mRNA, of duodenal Dmt-1 and Fpn-1 mRNA, and of duodenal ferroportin and DMT-1 protein, was quantitated by real-time PCR and by Western blotting, respectively.

**Results:** Iron homeostasis in both strains appeared normal until the animals were challenged by iron deficiency, when only the Gas/- mice developed severe anaemia. In iron-deficient Gas/- mice, massive (3-fold) splenomegaly was apparent together with an increased number of splenic megakaryocytes accompanied by thrombocytosis. Furthermore, a significant increase in erythropoietin concentration (16-fold) was observed in the Gas/- mice compared to the wild-type Balb/c mice on the low iron diet. The expression of the mRNA encoding the iron regulatory peptide hepcidin, Hamp, was down-regulated in both Cck2r/- and Gas/- mice on a low iron diet, but interestingly the reduction was greater in Cck2r/- mice and smaller in Gas/- mice than in the corresponding wild-type strains. The changes in Hamp mRNA expression were also reflected in Fpn-1 and Dmt-1 mRNA and protein concentrations. In the iron-deficient Cck2r/- mice the concentration of Fpn-1 mRNA was 1.9-fold lower than in the wild-type mice on the same diet.

**Conclusion:** These data suggest that gastrins play an important direct role, unrelated to their ability to stimulate acid secretion, in haematopoiesis under conditions of iron deficiency.


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Mutations of the same conserved glutamate residue in NBD2 of the SUR1 subunit of the KATP channel can result in either hyperinsulinism or neonatal diabetes

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Patients with neonatal diabetes (ND) or hyperinsulinemia (HI) often carry gain- or loss-of-function mutations in their KATP channels, respectively. KATP channels are octameric complexes composed of four SUR and four Kir6.2 subunits. Binding of ATP or ADP to Kir6.2 inhibits the channel whereas interaction of Mg-nucleotides with the nucleotide-binding domains (NBDs) of SUR stimulates channel activity. Mutation of a conserved glutamate (E1506) in the Walker B motif of NDB2 of SUR1 to lysine is known to cause HI by impairing Mg-nucleotide activation of the KATP channel. We now report two novel heterozygous SUR1 mutations (E1506D, E1506G) in separate patients with ND. To understand why mutations at the same residue can cause either ND or HI, we expressed wild-type (WT) and mutant channels in Xenopus oocytes and studied KATP currents with electrophysiological methods. Resting whole-cell KATP currents in oocytes expressing Kir6.2 and equal amounts of wild-type or ND mutant SUR1 (to simulate the heterozygous state) were larger than for WT channels. In pancreatic beta-cells, a similar increase in KATP current would impair insulin release, consistent with the diabetic phenotypes. The sensitivity of ND mutant channels to MgATP inhibition was slightly reduced: IC50 were 21±2μM (S.E., n=11), 19±2μM (n=10), 13±1μM (n=23) for homomeric E1506D, E1506G and WT, respectively. All mutations decreased channel activation by MgADP as assessed using WT or an ATP-insensitive Kir6.2 subunit, but had little effect on MgATP activation. Using wild-type Kir6.2, a 30s-preconditioning exposure to 10mM MgATP caused a marked reduction in the ATP sensitivity of ND channels (IC50=78±3μM (n=8) for E1506D; 108±10μM (n=7) for E1506G), a small decrease in that of wild-type channels (IC50=30±3μM, n=11) and no change for E1506K channels. Smaller shifts were observed with concentrations of MgATP >300μM. Thus differentially altered nucleotide regulation (MgADP for E1506K; MgATP for E1506G and E1506D) by NBD2 of SUR1 may explain the difference in resting whole-cell currents of ND and HI mutant channels and the respective clinical phenotypes.

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Adaptive metabolic response of whole body and skeletal muscle to periodic high sugar availability in lean sedentary individuals

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Hyperglycemia is a contributing factor for metabolic alterations seen in type 2 diabetes mellitus (T2DM). Skeletal muscle in T2DM displays reduced oxidative capacity with an elevation in glycolytic and lipogenic enzyme expression. Recently it was shown that transcription factors MondoA and MondoB mediate responses to elevated glucose availability in various cell types in vitro. Up-regulation of glycolytic and lipogenic gene expression in skeletal muscle was shown to be related to MondoA. While there is growing evidence about the regulatory impact of high glucose availability on metabolism in type 2 diabetes, less is known about the contribution of nutrition based periodic high glucose availability, like chronic ingestion of sugar sweetened beverages, in healthy sedentary individuals. Therefore we recruited sedentary healthy lean subjects without former history of chronic soft-drink consumption. They consumed sugar sweetened beverages (~2g sugar/kg body weight per day) on top of their usual diet for 4 weeks. Pre and post intervention fasting glucose, insulin and blood lipids were measured. Additionally muscle biopsies from quadriceps femoris were taken (under 1% lignocaine injection anaesthesia) for RT-PCR based metabolic gene expression measurements and Western Blot analysis of MondoA. Moreover, indirect calorimetry showed a significant increase in RER (Pre=0.75±0.09, Post=0.87±0.08) revealing a reduction in fatty acid oxidation and increase in carbohydrate metabolism, DEXA analysis revealed a gain in fat mass (Pre=15.2±5.1kg, Post=16.2±4.7kg). Fasting glucose was significantly increased (Pre=4.83±0.43mmol/L, Post=5.13±0.38mmol/L), while insulin levels (Pre=4.95±1.90μIU/L, Post=6.40±1.62μIU/L) and HOMA2 derived insulin resistance (Pre=0.68±0.19, Post=0.84±0.20) showed trends. Blood lipids did not alter post intervention. Muscle biopsy analysis revealed a significant increase in lipogenic ACC mRNA (Pre=0.95±0.52AU, Post=1.21±0.78AU) and glycolytic GAPDH mRNA (Pre=1.00±0.74AU, Post=1.94±1.52AU) expression, as well as a significant reduction of PGC1alpha mRNA (Pre=0.96±0.44AU, Post=0.79±0.53). Additionally, protein levels of MondoA (Pre=1.00±1.08AU, Post=2.58±2.46AU) showed a trend towards elevation post intervention. In conclusion 4 weeks sugar sweetened beverage consumption could shift substrate metabolism towards use of carbohydrate, increased fasting blood glucose and mediated alterations of metabolic gene expression in muscle towards increased glycolytic and lipogenic activity. In conclusion, we suggest that periodical high glucose availability, induced by repetitive soft-drink consumption, may contribute to alterations in metabolism known to be symptomatic for T2DM individuals even before the development of obesity.

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The metabolic syndrome represents a cluster of cardio-metabolic risk factors (1,2). It is suggested that this syndrome in adulthood has its origins in early development, where maternal obesity during pregnancy can confer increased susceptibility in the adult offspring (3,4). Most physiological processes follow a period of around 24h (termed ‘circadian’), and are regulated by endogenous timing system involving a set of ‘clock’ genes. Disrupting the circadian clock system leads to dysfunction of metabolic processes and the development of the metabolic syndrome (5). However, it is unknown whether high fat (HF) nutrition during early and late development can affect the clock system in the adult skeletal muscles. Therefore, we examined day-night expression of clock and clock-controlled genes in this key metabolic tissue. Female C57/BL6J mice were fed either a HF (45% kcal fat) or control chow (C, 21% kcal fat) diet prior to and during pregnancy and lactation. Weaned offspring were fed the HF or C diet, generating the dam-offspring dietary groups: C/C, C/HF, HF/C, HF/HF. Skeletal muscle tissue from 15-week old male offspring were taken 8h into the light and dark periods of a 12h light-12h dark light-dark schedule (n=5-6 per time point per treatment group), and gene transcript levels for the clock genes, BMAL1, CLOCK and PER2, and clock-controlled genes, Rev-Erbα, IRs1 and SIRT1, were determined using quantitative real-time PCR. BMAL1 expression was between 2.5 to 6.5 fold higher (p<0.05, t-test analysis) at night in the C/HF, HF/HF and C/C groups, respectively. This pattern was lost in HF/C offspring. CLOCK expression was 1.6 fold higher (p<0.05) at night in HF/HF, but this pattern was lost in the other offspring groups. In contrast, PER2 was 2.6 to 2.4 fold lower (p<0.05) at night in C/C and HF/HF, respectively, but this pattern was lost in C/HF and HF/C. Rev-Erbα was 2.1 fold and 1.8 fold lower (p<0.05) at night in C/C and HF/HF, respectively, but again no day-night changes were observed in C/HF and HF/C. ANOVA followed by post-hoc analysis showed that overall expression of BMAL1 was higher (p<0.05) in HF/HF vs. C/C, while PER2 was lower (p<0.05) in C/HF and HF/C vs. C/C. SIRT1 was lower (p<0.05) in C/HF, HF/C and HF/HF vs. C/C. IRs1 was higher (p<0.05) in HF/HF vs. C/C. Overall expression of CLOCK was similar in all treatment groups. The results suggest that maternal dietary fat intake alters day-night expression patterns of most of the clock and clock-controlled genes in offspring skeletal muscles, whilst extended HF exposure to adulthood alters overall expression level of most of these genes. Such changes could alter downstream metabolic processes, increasing susceptibility to the metabolic syndrome in adulthood.


Regulation of hepatic aquaporin-9 in starved rats is gender specific

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Aquaporin-9 (AQP9) is a broad-selectivity neutral solute channel that facilitates the hepatic uptake of glycerol. Once inside the cell, glycerol is phosphorylated by glycerol kinase (GlyK) into glycerol-3-phosphate, a precursor for gluconeogenesis and lipogenesis. In male rats, it is well established that hepatic AQP9 expression is increased in states with low plasma insulin levels such as starvation (1). In addition, in the fed state the hepatic abundance of AQP9 has been shown to be ~20% higher in male rats when compared to females (2). The aim of this study was to evaluate the influence of gender upon regulation of hepatic AQP9 expression in response to starvation. In contrast, starvation had no effect upon hepatic AQP9 expression in female rats. Coordinately, plasma glycerol levels remained unchanged in male rats whereas it was increased from 55±6 in the fed state to 112±15 μmol/l in starved females (p<0.05). In addition, biophysical assessment of hepatocyte membrane glycerol transport in starved rats showed higher permeability in males when compared to females. The hepatic GlyK expression was not affected by starvation, however, when comparing males with females in the fed state the relative abundance was 2.1±0.2 vs. 1.0 ±0.1, respectively (p<0.0001).

The effect of ovariectomy was investigated, to evaluate the role of female gender in the observed sexual dimorphism in hepatic AQP9 regulation. The animals were either ovariectomized (ovx) (n=12) or sham (n=6) operated under isoflurane anesthesia (induction: 4%, maintenance: 2% in atmospheric air) and treated with buprenorphine preoperatively (0.025 mg/kg i.p.) and postoperatively for 5 days (0.006 mg/ml in drinking water). Starvation of ovx rats (n=7) resulted in an increased expression of AQP9 similar to what was observed in starved males when compared to fed ovx rats (n=5), whereas GlyK abundance remained unchanged. Like in starved males, no increase in plasma glycerol was observed in the starved ovx females. Overall, these results demonstrate that sexual dimorphism exists in the initial hepatic handling of glycerol in starved rats. In addition, our data suggest that female gender is involved in preventing the starvation induced increase in hepatic AQP9 abundance observed in males.
Distinct responses to prostaglandin E2 (PGE2) by human myometrial smooth muscle cells isolated from the upper and lower segment of the pregnant human uterus

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A better understanding of the molecular pathways involved in the onset and maintenance of human labour could lead to improved management of preterm labour. Prostaglandins have long been implicated to play a role and our research has focused on the regulation of prostaglandin (PG) pathway in the uterus during pregnancy. Our overall hypothesis is the differential regulation of PG synthesis and PG receptors are important for maintaining the balance of myometrial quiescence throughout pregnancy and contractility during labour. We have demonstrated PGE2 can repress inflammatory chemokine output from primary human myometrial smooth muscle (MSM) cells isolated from lower segment of the uterus. This effect is mediated via EP2 and EP4, but not EP1 or EP3. As it has been suggested that there may be functional differences between upper and lower regions of the pregnant uterus, we wanted to investigate whether PGE2 would elicit the same effect in upper segment MSM cells. We hypothesize that MSM cells isolated from distinct uterine sites (upper vs lower segment) are capable of producing unique responses. Methods: Paired upper and lower segment myometrial biopsies were obtained from Caesarean section procedures at term, prior to labour onset and utilised to isolate RNA (n=12) or MSM cells (n=3). MSM cells were cultured to confluence and incubated over-night in serum free medium prior to treatment, with PG receptor agonists (10uM – 300pM) in the presence or absence of IL-1β (1ng/ml). Elaboration of interleukin-8 into the culture medium was assessed by ELISA. Results: Robust increases in IL-8 release by MSM cells from both the upper and lower segment of the pregnant uterus was observed following treatment with IL-1β compared to non-stimulated controls. In the lower segment MSM cells PGE2 significantly repressed IL-1β induced IL-8 output in a dose-dependent manner (Student’s t test, p<0.05 at 30nM); however, this PGE2-mediated repression was not observed in upper segment HMSCM cells. Furthermore, selective EP2 and EP4 agonists repressed IL-1β-induced IL-8 output in lower segment HMSCM cells but failed to do so in upper segment HMSCM cells. As it has been suggested that there may be functional differences between upper and lower regions of the pregnant uterus, we wanted to investigate whether PGE2 would elicit the same effect in upper segment MSM cells. We hypothesize that MSM cells isolated from distinct uterine sites (upper vs lower segment) are capable of producing unique responses. Methods: Paired upper and lower segment myometrial biopsies were obtained from Caesarean section procedures at term, prior to labour onset and utilised to isolate RNA (n=12) or MSM cells (n=3). MSM cells were cultured to confluence and incubated over-night in serum free medium prior to treatment, with PG receptor agonists (10uM – 300pM) in the presence or absence of IL-1β (1ng/ml). Elaboration of interleukin-8 into the culture medium was assessed by ELISA. Results: Robust increases in IL-8 release by MSM cells from both the upper and lower segment of the pregnant uterus was observed following treatment with IL-1β compared to non-stimulated controls. In the lower segment MSM cells PGE2 significantly repressed IL-1β induced IL-8 output in a dose-dependent manner (Student’s t test, p<0.05 at 30nM); however, this PGE2-mediated repression was not observed in upper segment HMSCM cells. Furthermore, selective EP2 and EP4 agonists repressed IL-1β-induced IL-8 output in lower segment HMSCM cells but failed to do so in upper segment HMSCM cells. In addition, a prostacyclin receptor agonist elicited repression of IL-8 in lower segment but not upper segment HMSCM cells.

Conclusions: We demonstrate that within the uterus, smooth muscle cells isolated from two distinct uterine sites are capable of producing unique responses. We intend to further characterize these cells in order to use them as a model to study different regions of the uterus and improve our understanding of PG signalling within the pregnant human uterus.

Astile S et al. [2007 Mol Hum Reprod 13(1):69-75

C58

Role of Rho-kinase and Src-Family Kinases in carbachol-induced contraction of isolated rat bronchioles

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Asthma is associated with remodelling of airway smooth muscle and hyper-responsiveness to constrictor stimuli. Potential therapeutic targets for the treatment of asthma are Rho-kinase and its principle activator RhoA. They have been implicated in both remodelling and constrictor hyper-responsiveness due to their involvement in smooth muscle migration, proliferation and constriction, the latter primarily through inhibition of myosin phosphatase (Ca2+-sensitization). The signalling pathways that control activity of RhoA/Rho-kinase however, such as the Src-family of tyrosine kinases (SrcFK), are largely unexplored.

We studied carbachol-induced contraction in isolated wire myograph-mounted rat bronchioles (1260± 68μm), bathed in bicarbonate-buffered physiological salt solution, pH 7.4 at 37°C, and investigated the effects of inhibitors of Rho-Kinase (Y27632, 10μM) and SrcFK (PP2, 30μM) on these contractile responses. Carbacho (0.001-100μM) caused a concentration-dependent constrictic (EC50 -5.84 ± 0.06 Log[M], n=18)), which was reproducible upon repetition (both EC50 and max unchanged, P>0.05, n=6). Y27632 significantly increased the EC50 (from -5.81 ± 0.09 to -5.32 ± 0.03, P<0.001, paired t-test, n=6) and decreased the maximal contractile response (by 17.3 ± 6.5%, p<0.05, paired t-test, n=6) (fig a). PP2 also significantly increased the EC50 (from -5.91 ± 0.10 to -5.64 ± 0.10, P<0.01, paired t-test, n=6) and decreased the maximal contractile response (by 10.1 ± 2.4%, p<0.01 paired t-test, n=6) (fig b). Carbachol also caused constriction in α-toxin permeabilised rat bronchioles with [Ca2+]i clamped at pCa 6.4 and in the presence of 10μM cyclopiazonic acid and 1μM GTP. This constriction was also inhibited by Y27632 (n=3). These results suggest the involvement of both Rho-kinase and SrcFK in contraction of rat bronchioles. We aim to further characterise these responses and determine whether SrcFK are acting upstream of Rho-kinase, as shown previously in pulmonary artery (1).

C59

Effect of purinergic and cholinergic costimulation on rat airway contractility

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Airway contractility plays a key role in obstructive respiratory dysfunctions. In airways, extracellullar ATP can be coreleased with ACh. In isolated airways, cholinergic stimulation induces a short-time contraction followed by a slow delayed contraction [1]. Extracellular ATP alone induces a transitory contractile response [2]. The aim of this study was to investigate the combined effect of extracellular ATP (10μM) and cholinergic stimulation on the dynamics of airway contraction. Isometric tension was measured on tracheal (T), extra- (EB) and intra-pulmonary (IB) bronchial rings from rats (Wistar, 8-15 week-old). The short-time phase was studied by simultaneous stimulation with ACh and low (0.3μM) and high (10μM) Carbachol (CCh) vs. CCh alone. Tension was expressed as % of reference (100%) and compared by Student’s t-test. The short-time phase was studied by applying ATP on CCh-precontracted airways. Indo-1 fluorimetry was used to determine [Ca2+]i peak and plateau values and, when occurring, oscillation frequency in freshly isolated T and EB. They suggest that ATP acts directly on airway contractility 

Effects of inhibitors of Rho-kinase and Src-family kinases on carbachol contraction in rat bronchioles

Smith et al. (2001) Biol Reprod 64: 1131-1137

Sparey et al. (1999) J Clin Endocrinol Metab 84: 1705-1710

Support by the Canadian Institute for Health Research (CIHR), the Alberta Heritage Foundation for Medical Research (AHFMR) and the Preterm Birth and Healthy Outcomes Team (PreHOT).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Effect of purinergic and cholinergic costimulation on rat airway contractility

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Airway contractility plays a key role in obstructive respiratory dysfunctions. In airways, extracellular ATP can be coreleased with ACh. In isolated airways, cholinergic stimulation induces a short-time contraction followed by a slow delayed contraction [1]. Extracellular ATP alone induces a transitory contractile response [2]. The aim of this study was to investigate the combined effect of extracellular ATP (10μM) and cholinergic stimulation on the dynamics of airway contraction. Isometric tension was measured on tracheal (T), extra- (EB) and intra-pulmonary (IB) bronchial rings from rats (Wistar, 8-15 week-old). The short-time phase was studied by simultaneous stimulation with ACh and low (0.3μM) and high (10μM) Carbachol (CCh) vs. CCh alone. Tension was expressed as % of reference (100%) and compared by Student’s t-test. The short-time phase was studied by applying ATP on CCh-precontracted airways. Indo-1 fluorimetry was used to determine [Ca2+]i peak and plateau values and, when occurring, oscillation frequency in freshly isolated T and EB. They suggest that ATP acts directly on airway contractility.
smooth muscle via P2X (in T and EP) and P2Y receptors (in EP and IB).


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C60
Rho-associated kinase-mediated modulation of Ca2+-signaling, myosin and MYPT1 phosphorylation, and force induced by membrane depolarization and carbachol of rat ureteric smooth muscle
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Ureteric peristalsis ensures the unidirectional flow of urine from the kidney to the bladder and is regulated by myogenic mechanisms and neurogenic factors. Unilateral ureteric obstruction of ureter in rabbits exhibited increased expression of both isoforms of ROKα and ROKβ, as well as enhanced contractile responses to different stimuli which were attenuated by the ROK inhibitor Y-27632. ROK inhibitors, therefore, represent a promising novel therapy for the prevention of renal colic-associated pain and kidney damage. In the present study, we examined the effects of ROK inhibition on Ca2+ signaling, LC20 phosphorylation, MYPT1 phosphorylation (at T697 and T855) and force activated by high [K+] induced depolarization (electromechanical coupling) and the carbachol (pharmacomechanical coupling) in rat ureteric smooth muscle. Rats (~200 g) were humanely killed using CO2 anaesthesia followed by cervical dislocation, in accordance with UK legislation. We used fast framing disc-based confocal imaging attached to a high sensitivity (iXon Andor) CCD camera, which allowed acquisition of images at 60-200 fps and thereby accurate measurement of temporal and spatial characteristics of Ca2+ signaling in intact ureteric strips. Inhibition of ROK activity decreased the amplitude of the plateau component of the action potential induced by high [K+], which resulted in a decrease in the amplitude of the phasic component of both the Ca2+-transient and force, but did not affect the rapid rise in LC20 phosphorylation. The sustained component of the K+-induced Ca2+-transient was reduced by ROK inhibition by 18.0 ± 0.5%, whereas LC20 was completely dephosphorylated and force returned to baseline. MYPT1 was found to be partially phosphorylated at both ROK sites under resting conditions. Phosphorylation increased only at T855 in response to K+, and ROK inhibition reduced the level of phosphorylation below that at rest. Inhibition of ROK also had no effect on Ca2+-transient, the rapid CCH-induced rise in LC20 phosphorylation or the initial rate of force development; however, it markedly increased the rate of LC20 dephosphorylation and relaxation. CCH induced MYPT1 phosphorylation at T855, but not T697, under these conditions. ROK inhibition prevented the CCH-induced increase in LC20 phosphorylation and reduced the basal level of phosphorylation at both sites. We conclude that ROK, acting via phosphorylation of MYPT1 at T855, inhibition of MLCP and increased LC20 phosphorylation, plays an important role in ureteric smooth muscle contraction.

British Heart Foundation
Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C61
Ca2+-activated Cl- channel expression and currents are upregulated by epidermal growth factor receptor activation in colonic epithelial cells
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Enhanced activation of the epidermal growth factor receptor (EGFR) occurs in a number of intestinal disorders associated with dysregulated epithelial transport, including infectious diseases and inflammatory bowel diseases. Furthermore, recent studies have implicated the novel Ca2+-dependent Cl- channel (CaCC), TMEM16A, in the pathogenesis of infectious diarrhoea. We have previously shown that EGF chronically potentiates colonic epithelial secretory function, an effect which in vivo would contribute to the onset of diarrhoea. Thus, in the present study we investigated a potential role for CaCCs in mediating chronic, prosecretory effects of EGF. T84 cell monolayers were mounted in Ussing chambers and the activity of transport proteins was measured as changes in short-circuit current. Protein expression/phosphorylation was measured by RTPCR and western blotting. Data are presented as mean ± SEM and were statistically analysed by ANOVA or Student's t-tests as appropriate. As previously reported, acute treatment with EGF (100 ng/ml; 15 min) increased Cl- secretory responses to the Ca2+-dependent agonist, carbachol (CCh; 100 μM) by 187 ± 15% (n = 53; p < 0.001) when measured 24 h later. Acute treatment with EGF did not chronically alter CCh-induced mobilization of intracellular Ca2+ (n = 9). Under conditions that isolate apical Cl- currents through CaCC, EGF potentiated CCh-induced responses to 173 ± 25% of those in control cells (n = 26; p < 0.01). Furthermore, we found that carbachol and protein expression of the CaCC, transmembrane protein 16A (TMEM16A), was increased by EGF to 256 ± 38% (n = 7; p < 0.01) and 297 ± 46% (n = 9; p < 0.001) of control levels, respectively. EGF-potentiation of Cl- secretory responses was inhibited by the general protein kinase C inhibitor, GF109203X (5 μM), but were unaltered by the PKCδ inhibitor, Go6976 (1 μM) and the PKCe translocation inhibitor (200 μM) (n = 5). In contrast, EGF-induced increases in CaCC currents and TMEM16A expression (n = 4) were significantly reduced in the presence of the PKCs inhibitor, rottlerin (20 μM). Furthermore, EGF significantly increased phosphorylation of PKCθ (n = 10), but not PKCα (n = 3), with a maximal effect occurring 1 h after treatment. Finally, the P13-K inhibitor, LY290042 (25 μM) inhibited the effects of EGF on Cl- secretion (n = 6), CaCC currents (n = 7), TMEM16A expression (n = 5) and PKCθ activation (n = 5). Ca2+-dependent Cl- conductances, likely mediated by TMEM16A, are chronically upregulated in response to EGFR activation in colonic epithelial cells through a mechanism involving PKCθ and PI3-K. Further elucidation of the molecular mechanisms involved may yield new targets for development of drugs to treat epithelial transport disorders.

Supported by Science Foundation Ireland Principal Investigator Award to SJK
Location of the ATP-dependent gate in the cystic fibrosis transmembrane conductance regulator channel pore

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Opening and closing of the cystic fibrosis transmembrane conductance regulator (CFTR) C1 channel is controlled by ATP binding and hydrolysis by its nucleotide binding domains (NBDs). This is presumed to lead to opening of a single “gate” within the permeation pathway, however, the location of such a gate has not been described. Recently our group described the presence of a “barrier” within the inactive channel pore that prevents access from the cytoplasm to the narrow pore region (1). Here we present evidence that this barrier reflects the location of the channel gate. We used inside-out patch clamp recording from the BHK cell line to monitor access of cytosolic cysteine reactive reagents (MTSES and MTSET) to reporter cysteines introduced at different sites in a cysteine-less CFTR pore, located in transmembrane (TM) regions TM1 and TM6.

To investigate the relationship between access to these sites and ATP-dependent channel gating, we used pharmacological and mutagenic approaches to manipulate NBD function. Addition of 2 mM pyrophosphate (Pi) to inhibit ATP hydrolysis, promote locking in the open state and increase overall channel open probability significantly increased the rate of modification by both MTSES and MTSET at Q98C (TM1) and I344C (TM6) (p<0.05) but had no effect on the rate of modification at K95C (TM1) or V345C (TM6) (p>0.4). We also manipulated NBD function by introducing the mutations K464A (which decreases channel opening rate and open probability) and E1371Q (which slows channel closure and increases open probability). The E1371Q mutation significantly increased the rate of MTSES modification at Q98C and I344C (p<0.02) but had no effect on the rate of modification at K95C or V345C (p>0.25). The K464A mutation significantly decreased the rate of MTSET modification at Q98C and I344C (p<0.005) but had no effect on the rate of modification at K95C or V345C (p>0.5).

These results therefore suggest that altering NBD function pharmacologically (using Pi) or non-pharmacologically (by mutagenesis) reveals a positive relationship between channel open probability and accessibility of residues in TM1 and TM6.


Supported by the Canadian Institutes of Health Research and Cystic Fibrosis Canada.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C62

C63

Thermodynamic mutant cycles suggest no interface separation during channel gating at CFTR’s non-canonical ATP binding site

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CFTR is an anion selective channel that plays an essential role in epithelial physiology. It is a member of the ATP Binding Cassette transporter superfamily. A conserved mechanism involving ATP binding and hydrolysis at cystolic nucleotide binding domains (NBDs) drives both gating of the CFTR channel and transmembrane substrate translocation by transporters. NBDs can dimerize, and two composite ATP binding sites are formed at the interface. In CFTR and its closest relatives (medically relevant sulphonylurea receptors and multi drug resistance-associated proteins) only one of the two composite sites is catalytically active, while the other, site 1, is non-canonical, and binds ATP tightly but does not hydrolyze it. At site 2, ATP binding and hydrolysis have been linked to localized closing/dehydration and opening/hydration, respectively, of the NBD dimer interface. In turn these are coupled to CFTR channel opening and closing. However, little is known about movements around composite site 1 associated with gating.

We used thermodynamic mutant cycles [1] to study the dynamics of the NBD dimer interface around composite site 1. T460 is a conserved residue in the NBD1 face of site 1. In homology models of CFTR, three residues (H1348, L1353 and H1375) on the NBD2 face of site 1 are close enough to interact with T460. In addition, the three positions yielded high correlations scores with T460, when multiple sequence alignments were analyzed to identify coevolving positions [2, 3]. We studied hydrolytic and non-hydrolytic channel gating in single mutants (T460S, L1353M, H1348A, H1375A) and double mutants (T460S/L1353M, T460S/H1348A, T460S/H1375A). Mutation T460S caused a small acceleration of hydrolytic and non-hydrolytic closure. Mutation L1353M had very little effect. Mutations H1348A and H1375A dramatically slowed hydrolytic and non-hydrolytic closure. Mutation H1353M had very little effect. The corresponding double mutants changes due to mutations at the NBD2 sites proved mostly additive with those caused by mutation T460S. Overall, changes in coupling energy (ΔΔGint = 0.43±0.14 kT, n=6, p=0.01 in non-hydrolytic closing for T460S/H1348A pair; mean±SEM, significance by t-test).

It is likely (as suggested by homology models and coevolution analysis) that at least one of the residues tested here interacts with T460. If two residues interact, small or non-significant changes in energetic coupling throughout the gating cycle signify only limited relative movement. Therefore these results support a gating model in which ATP-bound composite site 1 remains closed throughout the gating cycle.


Membrane expression of deltaF508 mutant increases epithelial HCO₃⁻ secretion via functional Cl⁻/HCO₃⁻ exchanger(s)

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Background: The CF-associated HCO₃⁻ secretion defect interferes with mucus clearance, nutrient absorption and fertility(1). "Corrector" drug therapy allows the most frequent mutation in CF, the delF508 mutant, to partially escape proteasom degradation and reach the apical membrane. But does this restore the HCO₃⁻ secretory defect? Aims: To find out whether, and by what molecular mechanism, delF508 membrane expression can promote epithelial HCO₃⁻ secretion in mouse intestine. Method and results: Basal and forskolin (FSK) stimulated duodenal HCO₃⁻ secretion was studied in vivo in luminally perfused (duodenum and colon) mice, which were anesthetized by spontaneous inhalation of isoflurane (a mixture of 2.2±0.2% isoflurane in |#65374|30-40% oxygen, |#65374|60-70% nitrogen) and were kept in physiological conditions following standard experimental procedure as previously described(2), as well as in vitro in isolated duodenal and colonic mucosa of CFTR KO(2), delF508 mutant(3), and WT littermates, congenic on FVB/N background. Western blot analysis was performed in isolated brush border membranes (BBM) and demonstrated approx 5%, and 0% glycosylated band C CFTR in the intestinal BBM of delF508, and CFTR KO, respectively, compared to WT BBM. The FSK-induced, CFTR dependent Isc response in the delF508 mutant intestinal mucosa was also approx. 5-10% of that in WT mucosa, whereas the CFTR-dependent HCO₃⁻ secretory response in delF508 was almost half of the WT response. Similar results were obtained in vivo. This markedly higher HCO₃⁻ than Isc response in the delF508 intestine was completely dependent on the presence of luminal CI both in the duodenum as well as colonic mucosa and can be inhibited by CFTR inhibitors Crftr(inh)-172(4) (20μM) together with GlyH101(5) (10μM). Conclusion: The data suggest that even a very low CI membrane conductance significantly enhances apical CI/HCO₃⁻ exchange. This suggests that delF508 "corrector" therapy has the potential to significantly enhance epithelial HCO₃⁻ secretion.


Disruption of clc-5 leads to a redistribution of annexin A2 in cells of the collecting duct and distal convoluted tubule of a Dent’s disease mouse model

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Mutations in CLCN5, encoding the voltage-dependent Cl⁻/H+ antiporter, CLC5, cause Dent’s disease (Type 1) characterised by low molecular weight proteinuria, hypercalciuria, and nephrolithiasis. Impairment of endocytosis occurs primarily in the proximal tubule, but CLC5 is also expressed more distally in the collecting duct (CD) intercalated cells. Expression of clc-5 occurs in several distal/collecting duct mouse cell lines such as mIMCD-3 and mpkDCT cells. In mIMCD-3 cells, clc-5 ablation results in defective endocytosis and plasma membrane expression of the crystal adhesion molecule, annexin A2 together with crystal agglomeration. Antisense clc-5 treatment of the mpkDCT cell model of the distal convoluted tubule (DCT) disrupts endocytosis. Here we investigate the effect of clc-5 disruption on annexin A2 distribution in mpkDCT cells. We extend this to examine annexin A2 localisation in the CD and DCT of the Guggino clcn5 knockout mouse, a model that recapitulates the renal attributes of Dent’s disease, including intra-tubular Ca²⁺-crystal deposition. Using immunocytochemistry and confocal microscopy, annexin A2 redistribution was determined in mpkDCT cells transfected with control GFP (transfection marker) and in cells where endogenous clc-5 was disrupted through cotransfection with antisense clc-5 and GFP². Paraffin embedded sections of kidney tissue of wild type (WT) and clcn5 knockout (KO) mice (12 months, high citrate diet) were subjected to antigen retrieval with 0.8M urea and assessed for annexin A2 distribution. Annexin A2 was detected by a rabbit anti-annexin A2 antibody (H-50 raised against residues 1–50 of human protein, cross-reactive with mouse) at 1:100 (Santa Cruz). Goat anti-aquaporin 2 antibody (epitope mapping at the C-terminus, human origin, cross-reactive with mouse, 1:250) and goat anti-calbindin antibody (epitope mapping at the C-terminus of Calbindin D28K, human origin, cross-reactive with mouse, 1:100) were used as markers of the CD and DCT respectively. Appropriate secondary antibodies were applied. Control GFP transfected mpkDCT cells showed an intracellular perinuclear location for annexin A2 (n=6). Following transfection with antisense clc-5, there was a marked redistribution of cytoplasmic annexin A2 to the cell periphery (n=5). In the WT mouse, annexin A2 showed an intracellular, vesicular pat-
tern in cell types within the DCT and CD. In the clcn5 knock-out, annexin A2 relocalised to the apical (lumen) cell pole in cells within the DCT and CD. All experiments were carried out in material from 3 KO or WT animals. We speculate that abnormal expression of the crystal binding molecule annexin A2 at the cell surface, together with hypercalciuria, will facilitate intra-tubular Ca\(^{2+}\)-crystal retention within the collecting of KO animals.


Supported by KRUK & NCKRF

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**C66**

Direct G protein modulation of K\(_{\text{G}}\) channel TASK-2: a role for basic residues in the C-terminus domain

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K\(_{\text{G}}\) TASK2 K+ channels have been shown to underlie \(I_{\text{k,vol}}\), the cell volume regulated K\(^+\) conductance involved in regulatory volume decrease, RVD, in Ehrlich cells and proximal tubule cells (Niemeyer et al., 2001; Barrière et al., 2003). RVD is the process in which cells swollen in hypertonicity recover their volume by activation of \(K^+\) and Cl\(^-\) channels followed by KCl efflux and osmotically-obliged water. Using Ehrlich cells we have also shown that \(I_{\text{k,vol}}\) is tonically inhibited by an active G protein and that this inhibition is relieved by cell swelling and G protein inactivation (Niemeyer et al., 2002). We now show results that suggest that TASK2 channels are modulated by G\(_{\beta\gamma}\) subunits of heterotrimeric G protein.

In whole cell patch-clamp studies of TASK2 expressed in HEK-293 cells with 100 \(\mu\)M GTP and 1 mM MgATP, current became stable at 60 ± 4 % (SEM, n=9) of initial value at >5 min after the onset of the recording. When GTP-\(\gamma\)S was used as a replacement for GTP there was a strong inhibition of TASK2 currents (15 ± 2 %, n=12), but if GDP-\(\beta\)S was used instead no inhibition was seen (79 ± 5 %, n=7). Intracellular addition of purified G\(_{\beta\gamma}\) inhibited TASK2 (27 ± 4 %, n=7) current, independently of whether GTP or GDP-\(\beta\)S was present. The effects of GTP-\(\gamma\)S and G\(_{\beta\gamma}\) were abolished by neutralisation of TASK-2 C-terminus double lysine residues K257-K258 or K296-K297.

Tagged proteins and immunoprecipitation assays were used to see if there is a physical interaction between G\(_{\beta\gamma}\) subunits and TASK-2. TASK-2 was seen to associate with G\(_{\beta\gamma}\) only (n=6), in agreement with the highest expression of the subunits in proximal tubule cells. Immunoprecipitation was impeded by mutating Cterminus K257-K258 (but not K296-K297) to alanines (n=4 for each mutant).

Finally we investigated whether the G protein effect might be involved in the extra and intracellular pH-gating of TASK-2. Gating by extra- or intracellular pH was unaltered in GTP-\(\gamma\)S-insensitive TASK-2-K257A-K258A mutant. Shrinking TASK-2-expressing HEK-293 cells hypertonic, 400 mOsm solution decreased the current to 0.36 ± 0.03 (n=7) of its initial value. The same manoeuvre had a significantly diminished effect on TASK-2-K257A-K258A-expressing cells, with a decrease in current to only 0.71 ± 0.03 (n=10) of the initial value.

Our data are compatible with the concept that TASK2 channels are modulated by G\(_{\beta\gamma}\) subunits of heterotrimeric G protein. We propose that this modulation is a novel way in which TASK-2 can be tuned to its physiological functions.

Barrière H et al. (2003). *J Gen Physiol* 122, 177-190


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Supported by Fondecyt grants 3085021 and 1090478. CECS is supported by Conicyt PFB and CIN by Conicyt and the Gobierno Regional de Los Ríos.

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**C67**

Ipsilateral corticospinal contributions to control of the forelimb in monkey

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Strong experimental evidence implicates the corticospinal tract in voluntary control of the contralateral forelimb. Its potential role in controlling the ipsilateral forelimb is less well understood, although anatomical projections to ipsilateral spinal circuits are identified. We investigated inputs to motoneurons innervating hand and forearm muscles from the ipsilateral corticospinal tract using multiple methods. Intracellular recordings were made from 62 motoneurons in three anaesthetized macaque monkeys. Monkeys were deeply anesthetised with sevoflurane (3–5% in 100% \(\text{O}_2\)) and alfentanil (7–23 \(\mu\)g kg\(^{-1}\) h\(^{-1}\)) by IV infusion during a laminectomy for exposure of spinal segments C6-T1. The anesthetic regime was then switched to an intravenous infusion of propofol (5–14 mg kg\(^{-1}\) h\(^{-1}\)) and alfentanil (doses as above). Neuromuscular blockade was achieved by infusion of atracurium (0.6-1.2 mg kg\(^{-1}\) h\(^{-1}\)). Continuously monitored vital signs included heart rate, arterial and venous blood pressure, blood oxygen saturation, end-tidal \(\text{CO}_2\), and core temperature. Depth of anaesthesia was verified by ensuring that there were no changes in heart rate or arterial blood pressure in response to peripheral nerve stimulation.

No monosynaptic post-synaptic potentials were observed following single and multiple shock stimulation of the ipsilateral corticospinal tract (300\(\mu\)A, Fig. 1). Single stimulus intracortical microstimulation of the primary motor cortex (M1) in two awake behaving monkeys (up to 30\(\mu\)A) failed to produce any responses in ipsilateral muscles. Strong stimulation (>500\(\mu\)A, single shock) of the majority of corticospinal axons at the medullary pyramids revealed only weak suppressions in ipsilateral muscles at longer latencies than the robust facilitations seen contralaterally (Fig. 2). Spike triggered averaging of ipsilateral muscle activity from M1 neural discharge (184 cells) did not reveal any post-spike effects consistent with monosynaptic corticomotoneuronal connections. We conclude that, in normal adults, any inputs to forelimb motoneurons from...
the ipsilateral corticospinal tract are likely to be weak and indirect.

Figure 1: A. Intracellular responses of forearm flexor motoneuron (MN) responding to single shock stimulation of contralateral corticospinal tract (cPT: gray trace) and ipsilateral corticospinal tract (iPT: black trace). Top two traces are intracellular recordings and bottom two traces are cord dorsum recordings. B. Same as A but multiple shocks given to iPT and cPT. C. Histogram showing the types of MNs tested with iPT/cPT, and maximum number of stimuli used. Bars to the right of the dotted line correspond to cPT stimulation to single shock. Gray bars indicate oligosynaptic responses, black bars monosynaptic responses.

Figure 2: Stimulus triggered averages of bilateral rectified muscle activity (Extensor Digitorum Communis: EDC, first Dorsal Interosseus: IDI), using left corticospinal tract electrical stimulation at intensities of 500 and 1000 μA (black & gray; number of stimuli: 1511 & 919 respectively). The arrows under each trace indicate the onset latency of the response of the right hand side muscle. The stimulation was given while the monkey was performing a bimanual precision grip task - this involved a 1s precision grip with either left hand only, right hand only, or both hands together. The trial type order (left hand, right hand or bimanual) was randomised.


Funded by the Wellcome Trust and the BBSRC.

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Oscillatory corticomuscular coupling during isometric voluntary contraction influences reaction time in humans

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It has been well documented that the sensorimotor cortex activity shows coherence with muscle activity within the 15–35 Hz band (beta-band) during weak to moderate isometric contractions (e.g. 1-3). Recently, we reported that subjects with greater coherence between the electroencephalogram (EEG) over the sensorimotor cortex and electromyogram (EMG) of contracting muscle show prominent grouped discharge in the EMG signal within the beta-band (4). Although it is possible that such grouped discharge may affect motor performance, its functional role in the control of human movement still remains unclear. The present study aimed to examine how beta-band oscillations in EMG influences reaction time. We recruited seven healthy human subjects (four males and three females, 21-26 years) who showed significant EEG-EMG coherence during a sustained isometric contraction of the tibialis anterior muscle in a preliminary experiment. Subjects first performed a steady contraction at 30% of maximal effort for 5-7 s, and then reacted to a sound cue by performing a ballistic dorsiflexion as quickly as possible. They repeated this task for 100 trials. Reaction time was measured from the cue to the time when the force signal exceeded the mean + 10 SD of data of measured over 1 s before the cue. We found that within each subject, trials could be divided into two groups: those where EMG showed prominent grouped discharge within the beta-band before reaction (GD+), or those where it did not (GD-). Pooled EEG-EMG coherence before the reaction was calculated in both groups; coherence was greater in GD+ than in GD-. This implies that even within the same subject, the strength of corticomuscular coupling changed from moment to moment, resulting in the difference in the degree of grouped discharge in EMG during the initial contraction. Further, in order to examine the effects of such grouped discharge in EMG on reaction time, we assessed the differences in reaction time between GD+ and GD- within each subject by using an unpaired t-test. The reaction time in GD+ was longer than that in GD-.
for all seven subjects, and was significantly so in three subjects
(Subject 1, GD+, 744 ± 49 ms, GD-, 722 ± 54 ms; Subject 4, GD+, 767 ± 44 ms, GD-, 748 ± 47 ms; Subject 6, GD+, 785 ± 59 ms, GD-, 757 ± 42 ms; P < 0.05). These findings suggest
that when grouped discharge develops in EMG during a steady
contraction, reaction time is delayed, entrained by the cycle
of oscillatory corticomuscular coupling.

Figure 1. Example reaction time difference between GD- and GD+.


The present study was supported by the Strategic Research
Program for Brain Sciences (SRPBS) from the Ministry of
Education, Culture, Sports, Science, and Technology (MEXT),
Japan.

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requirements.

C69

Extracellular spike duration in macaque motor cortex
pyramidal neurons is correlated with axonal conduction
velocity

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A number of recent studies have suggested that it may be pos-
sible to distinguish extracellular recordings of cortical inter-
neurons from pyramidal neurons on the basis of the relatively brief
spike duration in interneurons (1, 2). If this suggestion is cor-
rect, it would provide an important means of identifying cell
types in recordings from awake monkeys.

We investigated the spike duration of one class of pyramidal
neuron, namely corticospinal neurons physiologically identi-
fied via antidromic stimulation of the pyramidal tract using
pairs of implanted electrodes in 4 awake macaque monkeys,
prepared for recording under isoflurane anaesthesia (1.8-2.0%
in 50:50 O2 + N2O) (3). We first analysed the distribution of
antidromic latencies (ADLs) of 265 identified pyramidal tract
neurons (PTNs) recorded in primary motor cortex (M1, 172
PTNs, 4 animals) and ventral premotor cortex (PMv, area F5,
93 PTNs, 2 animals).

The distribution of M1 ADLs was skewed towards short ADLs
(0.5-5.6ms, median 1.1ms) and significantly different from
that of F5 ADLs, which was more symmetrical (median 2.5ms).
We found a few M1 PTNs with longer ADLs (>5ms) and some
F5 PTNs with ADLs>8ms, which equates to an axonal conduc-
tion velocity of <10m/s. These slow conducting PTNs are
known to far outnumber large ones but are much less stud-
ied due to recording bias.

We also investigated the distribution of the duration of extra-
cellularly recorded spike shapes defined as the time between
the trough to the next positive peak of the spike. The short-
est and longest duration spikes had widths of 150 and 750μs,
respectively. Interestingly, the distribution of F5 spike dura-
tion was bimodal, possibly suggesting different classes of PTNs
in F5 (3). In general we saw no significant differences in the
distribution of durations of identified PTNs vs. large spikes
recorded from other unidentified neurons (n=97).

Importantly, we found that there was a positive linear corre-
lation between ADL and spike duration in both M1 (R2=0.53,
p<0.001) and F5 (R2=0.52, p<0.001) (see Figure). Since the
ADL reflects axonal conduction velocity, this result means that
PTNs with the fastest axons (shortest ADLs) had the briefest
spikes. Further, since it is known that PTN soma size is corre-
lated with axon size and conduction velocity(4), it is likely that
the largest pyramidal neurons (Betz cells in M1) have spikes
with short durations (150-450μs) which overlap heavily with
those recorded for putative interneurons (430±270μs (1))
measured in the same way as in this study.

In summary, spike duration may not be a reliable indicator of
cell type, but more likely reflects discharge properties shared
between cortical interneurons and pyramidal neurons.

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requirements.
Effects of Area 3a stimulation on muscle activity in the awake monkey
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Rathelot and Strick (2006) reported that ~15% of corticomotoneuronal cells in macaques originate in area 3a instead of M1. They proposed that these cells contacted gamma motoneurons based on the lack of overt stimulation effects in area 3a (Widener and Cheney, 1997). In an ongoing study we are examining the effects of intra-cortical microstimulation (ICMS) in area 3a, area 3b and M1 in awake macaques. Two monkeys were trained to allow neck and arm restraint. These monkeys were implanted under general anaesthesia (3.0-5.0% sevoflurane and 0.025mg/kg/hr alfentanil) and aseptic conditions with a headpiece, to allow head fixation, and a recording chamber placed over the central sulcus. In the same surgery patch electrodes were placed on the surface of 4 forearm muscles to record electromyographic (EMG) activity. In daily experiments electrodes were inserted into sensorimotor cortex using a microdrive. Different areas were identified based on location and receptive field testing. In some sessions surface EMG electrodes were used to record first dorsal interosseus (1DI) activity. The effects of single pulse and multiple pulse ICMS (5-70 μA, 100 ms pulse width) on resting EMG were examined.

Preliminary results suggest that Area 3a sites are capable of generating overt twitches with multiple pulse ICMS. In the small number of sites so far examined, the ICMS effect closely corresponded to the receptive field of the cells at that site (for example ICMS at a site which responded to tapping the belly of the 1DI muscle produced twitches of 1DI at 20 μA). M1 sites close by generally had lower threshold ICMS effects but diffuse cutaneous receptive fields on the hand. Area 3b sites had clear focal cutaneous receptive fields on the digits but no clear ICMS effects. The latencies between the stimulus and the EMG response were similar for both M1 and Area 3a. These results suggest that, contrary to earlier studies, Area 3a is capable of activating extrafusal muscle fibres. Since the latencies of the area 3a and M1 responses were similar, it is plausible that the area 3a effect is mediated via corticomotoneuronal cells in area 3a. These could contact either alpha or beta motoneurons.


Funded by The Wellcome Trust.

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On the origin of sense of force and weight
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As a muscle is weakened by fatigue or partial paralysis, the increase in the motor command needed to lift a weight is thought to explain the increasing subjective heaviness of the lifted object (1). In these studies we show that peripheral signals normally underlie this sense of exerted force. With different fatiguing contractions we halved the force output of the thumb flexor muscles, which were then used to lift an object. For normal subjects, this resulted in objects feeling the same or lighter, consistent with the expected effects of the conditioning contractions on the sensitivity of peripheral receptors and contrary to the common view that perceived heaviness would increase. In contrast, for two deafferented subjects the perceived heaviness of the lifted object approximately doubled, in keeping with the theory of a central-signal associated with the motor command. In another experiment we completely paralysed the forearm muscles with curare and then allowed them to recover to half of their force output. This resulted in objects feeling lighter when lifted by the semi-paralysed thumb, even though the motor command to the motoneurones must have been greater. This is readily explained by reduced peripheral reafference associated with the lift, caused by the prolonged paralysis of muscle spindle intrafusal fibres. We conclude that peripheral signals, including a major contribution from muscle spindles, give rise to the sense of exerted force. In concept, however, reafference from peripheral receptors may also be considered a centrally generated signal. These results therefore challenge the distinction between central- and peripheral-based perception, and the concept that muscle spindles contribute only to perception of limb position and movement.


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Do muscle afferents contribute to the sense of body ownership?
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The sense of body ownership, a sense of which things ‘belong’ to our body, is presumably generated from sensory information. Both touch and proprioception seem ideal for this as they only signal events that occur on or within the body. The sense of body ownership can be manipulated using tactile stimuli to induce an illusion of ownership over a rubber hand (Botvinick & Cohen, 1998). When they experience this illusion, subjects
feel touch on the rubber hand, report that the rubber hand is their hand, and they have a physiological response to threats against the rubber hand. It is not known if proprionceptive signals from muscle receptors can manipulate the sense of body ownership in a similar way. We developed a novel illusion of ownership over a plastic finger, using movement of the index finger as the stimulus. The subject’s finger, which was hidden from the subject, was moved about the proximal interphalangeal (PIP) joint while the subject observed a plastic finger making movements identical to those of their finger. When the illusion was established, naïve subjects (n=30) reported that they felt the movement at the location where they saw the plastic finger and that the plastic finger was their finger. This illusion was induced after a digital nerve block (lidocaine 1%, 3-6 ml), which removes input from skin and joint receptors but leaves intact the muscle receptors in the long flexor and extensor muscles of the fingers. We also measured the impact of the illusion on the perceived position of the finger. Subjects (n=10) were shown a ruler with numbered graduations and asked to identify the number to which their finger was pointing. The plastic finger was 12 cm above the subject’s finger. In a control condition, subjects were accurate with this judgement and reported that their finger was 11.5 [10, 13] cm (median [IQR]) above the table, but after the illusion of ownership over the plastic finger was established, they reported that their finger was 19 [18, 21] cm above the table. This difference was significant (p < 0.005). These results show that proprioceptive signals from muscle receptors can contribute to the sense of body ownership. Furthermore, this illusion of ownership biases the perception of the position of the finger in space.


National Health and Medical Research Council.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C73

Alveolar epithelial CNGA1 channels mediate cGMP-stimulated, amiloride-insensitive lung liquid absorption

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Impairment in lung liquid absorption can lead to severe respiratory symptoms such as those observed in pulmonary oedema (1). In the adult lung two principle pathways are involved in cation transport which drives liquid absorption; a well established amiloride-sensitive, Na+ channel (ENaC) (2,3), and more controversially, an age-dependent, amiloride-insensitive pathway which has been suggested to be mediated by cyclicnucleotide gated (CNG) channels (4). We set out to elucidate the exact molecular identity of this channel and its physiological role in lung liquid absorption. Using immunohistochemistry we show that CNGA1, but not CNGA2 or CNGA3 channel subunits are expressed in the rat lung alveolar epithelium, predominantly in type I pneumocytes. We also developed an in situ assay of lung liquid absorption, briefly, male Wistar rats were given a terminal anaesthetic (i.p. injection of 1:1 ratio of hypnorp and hypnovel) and ventilated whilst the chest was opened and the pulmonary circulation was perfused. The lungs were then instilled with a liquid containing an impermeant tracer which the concentration of which was calculated in later samples to calculate changes in liquid volume, and thus rate of lung liquid absorption (Jv) during control and drug application periods. Application of 1mM 8Br-cGMP increased significantly Jv from -0.92±0.12 ml.h⁻¹.g⁻¹ to -1.35±0.09 ml.h⁻¹.g⁻¹ (n=10; P<0.01). When 8Br-cGMP was applied in the presence of 100mM 1-cis-diltiazem or 100mM pseudecdotoxin (PsTx), a specific toxin inhibitor of CNGA1 channels there was no significant difference between control and test periods; 0.86±0.03 ml.h⁻¹.g⁻¹ (control) vs. -1.00±0.09 ml.h⁻¹.g⁻¹ (1mM 8Br-cGMP + 100mM 1-cis-diltiazem; n=4; P>0.1) and -1.21±0.16 ml.h⁻¹.g⁻¹ (control) vs. -1.13±0.10 ml.h⁻¹.g⁻¹ (1mM 8Br-cGMP + 100mM PsTx; n=4; P>0.5). Using heterologous recombination of CNGA1 and αβγ-ENaC channels in HEK293 cells we showed that 100mM PsTx had no effect on ENaC channels and but did inhibit CNGA1 currents. We also showed that Amiloride had no effect on CNGA1 channels suggesting there is no pharmacological overlap between these channels. In situ, importantly 8Br-cGMP was able to stimulate some lung liquid absorption even in the presence of a maximal inhibitory dose of amiloride (50μM). Furthermore the CNGA1 channel antagonists had little effect on terbutaline-stimulated lung liquid absorption, which could be completely ablated by amiloride. Taken together, these results confirm the importance of CNG channels in cGMP stimulated lung liquid absorption, show the molecular identity of these channels to be CNGA1, further highlight the likely importance of alveolar type I pneumocytes in lung liquid regulation and reinforce the idea that there are two non-overlapping pathways by which liquid absorption can be stimulated in the adult lung and thus may be targeted in the treatment of diseases characterised by lung liquid overload.


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C74

The effect of ageing on the control of breathing: a role for the development of obstructive sleep apnoea

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Obstructive sleep apnoea (OSA) is a condition characterised by the frequent collapse of the upper airway during sleep. OSA is common among the general population, with one in fifteen adults presenting with moderate to severe OSA, but is much more prevalent among older individuals, affecting almost half of those aged over 60. One suggested mechanism for this striking prevalence in the elderly population is a change in the sensitivity of the control of breathing during sleep. (Younes, Ostrowski et al. 2001; Wellman, Malhotra et al. 2007).
Existing research on the causal properties of chemical drive in the development and/or progression of OSA do not take into account the alterations in chemical drive brought about by OSA itself and chronic changes in ventilatory control that may persist post-treatment. Furthermore, most studies use inadequate re-breathing methods for eliciting ventilatory responses and/or the choice of middle-aged participants is flawed since this is not the population most at risk of OSA development. In the present study, our main aim was to investigate the effect of ageing on chemosensitivity by measuring the ventilatory responses of young (18-30 years) and old (60-75 years) participants. A second aim was to investigate the differences in ventilatory control in the older population, between those who were free of sleep disorders (non-snorers and apnea-hypopnea index (AHI) = 0), and those suffering from very mild sleep disorders (snorers and AHI<10). The presence of any form of sleep apnoea and/or snoring was tested using at home testing equipment (ResMed Apnealink). This allowed us to map the role of chemosensitivity in the evolution of sleep disorders in the elderly population.

To evaluate chemosensitivity we used a hypercapnia multi-frequency-binary sequence (MFBS) challenge (Pedersen, Fatemian et al. 1999). A MFBS test involves a number of hypercapnic steps (10mmHg+resting PCO2) of varying duration. The hypercapnic steps were achieved using a dynamic end-tidal forcing system. PCO2 was controlled at 100mmHg throughout the test. Using the MFBS test the contributions of peripheral and central chemoreceptors in ventilatory control can be assessed. Preliminary results showed a 15.3±6% reduction in the combined sensitivity of peripheral & central chemoreceptors when older healthy participants were compared to younger healthy individuals. In addition, when older participants with very mild symptoms of sleep disorder were compared with asymptomatic age-matched individuals, a 9.2±2% reduction in combined sensitivity was found. These results suggest a reduction in chemosensitivity with age. When this attenuation becomes too low it could play a role in the development of sleep disordered breathing.


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C75

Interrelationship between conduction velocity, intracellular Ca2+ and gap junction resistance in ventricular myocardium

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Arrhythmogenesis could originate from local alteration in action potential (AP) conduction caused by changes to gap junction resistance (Rj). Cardiac arrhythmias are associated with a rise of intracellular Ca2+ concentration ([Ca2+]i) and an increased activity of Ca2+-calmodulin dependent protein phosphatase, calcineurin (CaN). Rj is a function of [Ca2+]i, which increases during rapid pacing, and the level of connexion 43 (Cx43) phosphorylation. This study aims to investigate the association between CV, [Ca2+]i, and Rj and role of CaN as a mediator.

Guinea pig left ventricular papillary muscles were superfused with control Tyrode’s solution at 37°C and point stimulated to generate propagated APs, recorded at known distances, d, by 3 M KCl-filled microelectrodes and conduction delay, t, recorded. Conduction velocity, CV, was calculated as d/t. Rj was measured from recordings of tissue impedance, Z (30 Hz-300 kHz), in an oil-gap bath to constrain current flow to the intracellular space. Total and dephosphorylated Cx43 protein expression was measured by Western blot. [Ca2+]i was increased by: 1) rapid pacing (1 to 5Hz) and 2) low Na (29 mM Na) containing Tyrode’s solution. Data are expressed as mean±SD/SE, significance (p<0.05) by Student’s t-test or one-way ANOVA.

Rapid pacing reduced CV from 47.4±2.1 to 33.7±2.9 cm/s (p<0.001; n=12). Similarly, low Na Tyrode slowed CV to 64.3±11.4% of control (p<0.05; n=4). Pretreatment with the calcineurin inhibitor, cyclosporine A (CysA; 10 μM) reversed the slowing in completely the slowing of CV at 5 Hz (p<0.001) and partially with low Na solution (80.0±5.7% of control; p<0.05; n=4). The latter intervention increased Rj (360±86.9 to 687±85; p<0.01; n=6) as well as the expression of dephosphorylated Cx43 (n=3). This was not accompanied by any change in the total Cx43 protein expression. Furthermore, the gap junction uncoupler, carbamoloxime (20μM; acts via dephosphorylating Cx43) slowed CV (70.9±1.3 to 59.2±3.1; p<0.05; n=6) and increased Rj (393±51 to 502±80; p<0.05; n=6). In summary, slowing of CV, which was reversed by CysA, was mainly due to an increase in Rj. We propose this is due to calcineurin-induced dephosphorylation of the gap junction protein Cx43, due to raised [Ca2+]i.

Funded by British Heart Foundation.

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C76

Mapping of cardiac activation with electrode arrays of monophasic action potential in vivo and in vitro

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Background — Optical mapping has been used to study action potential and its propagation for long time. However, it cannot be utilized in vivo and/or in working heart due to its technical limitations such as the application of excitation-contraction uncoupler and its side effects. In general, excitation-contraction uncoupler (for example, BDM or Cytochalasin D) not only stops normal sinus rhythm and heart beating, but also causes abnormal action potential and conduction (Cheng et al. 2004; Hayashi et al. 2003).

Methods and Results — We have currently developed a multi-electrode array system for mapping of monophasic action potential (MAP). The system consisted of two 9×9 MAP-electrode arrays (0.5×0.5 and/or 1×1 cm) and computerized data acquisition and analysis, which was able to synchronously record the MAPs at two regions in vivo and/or in working heart perfused physiologically in vitro and in situ (Hao et al. 2009).

A catheter electrode array of MAP was also developed, which was successfully used to record the MAPs in canine hearts. The
animal protocols were in accordance with Home Office regulations and Chinese rules. Adult rats were stunned and killed by the cervical dislocation, whereas adult rabbits were killed with an overdose of pentobarbitone. Adult canines were anesthetized with morphine (2 mg/kg) and alpha chloralose (100 mg/kg) and ventilated by a respirator. A recording sample from one MAP array is shown in the figure.

Conclusions — The main advantage of this system is that mapping of cardiac activation can be carried out in normal working heart. Based on the application of catheter MAP electrode array, it is also considerable and feasible in clinical studies and practices.

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Figure 1. A map of MAPs between superior vena cava and right pulmonary veins in the rat heart perfused physiologically in vitro


This work was supported by British Heart Foundation & National Natural Science Foundation of China.

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Integrated assessment of cardiac contractility and calcium/calcmodulin protein kinase II delta expression and activity following acute and chronic isoprenaline administration

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Calcium/calcmodulin protein kinase II delta (CaMKIIδ) plays a central role in normal cardiac calcium handling and contractility and has also been identified as a molecular switch, triggering contractile dysfunction in cardiomyopathy. As such, it may be an important intracellular target for drugs that alter cardiac performance and could be a useful parameter to measure in cardiac pharmacological safety studies. Here we present data showing the effects of isoprenaline on cardiac contractility and CaMKIIδ expression and activation in male Dunkin-Hartley guinea pigs (GPs) following acute and chronic administration.

In acute experiments, contractility was assessed by measurement of left ventricular (LV) dP/dtmax. GPs (430-640g) were anesthetised with fentanyl (50 μg kg⁻¹ s.c.) followed by sodium pentobarbital (50-60 mg kg⁻¹ i.p.) and a Millar Tip catheter was placed in the lumen of the LV. Cumulative doses of isoprenaline (Iso; 0.1, 0.3, 1.0 nmol kg⁻¹ min⁻¹) or vehicle (saline, Sal) were infused i.v. for 15 min each dose (n=4). LV dP/dtmax was increased by Iso (3325 ± 288 to 8500 ± 591 mmHg s⁻¹, p<0.05, one way ANOVA plus Dunnett’s test) while Sal had no effect (3415 ± 487 to 3536 ± 155 mmHg s⁻¹). In chronic experiments, contractility was assessed by echocardiography. GPs (450-650g) were anaesthetised with 6 mLkg⁻¹ Hypnorm/Hypnovel. Iso (1.5 μmol kg⁻¹ day⁻¹, n=4) or vehicle (acidified saline, AS; n = 2) was delivered via a minipump for 6 days. AS did not alter LV diastolic diameter (LVDD) and fractional shortening (FS) (6.4 ± 0.6 to 6.6 ± 0.3 mm and 68 ± 5 to 75 ± 7 %, respectively) while LV systolic diameter (LVSD) decreased (2.0 ± 0.5 to 1.6 ± 0.4 mm, p<0.05, paired t-test). Iso increased both LVDD and LVSD (5.8 ± 0.6 to 7.1 ± 0.3 mm and 2.0 ± 0.4 to 3.8 ± 0.2 mm, respectively, p<0.05) while FS decreased (67 ± 4 to 46 ± 2 %, p<0.05).

Quantitative immunoblotting was performed to assess total CaMKIIδ protein levels in GP LV homogenates. Following acute Iso no changes were seen in expression, however, chronic Iso increased CaMKIIδ expression to 1.01 ± 0.03 vs 0.63 ± 0.07 in AS (p<0.05, unpaired t-test). CaMKII activity was assessed by incorporation of γ³²P into a CaMKII peptide substrate, autocamtide. Acute Iso increased CaMKII activity to 2.5 ± 0.32 compared with 1.9 ± 0.13 in Sal (p<0.05, unpaired t-test), while chronic Iso increased CaMKII activity to 1.89 ± 0.10 vs 1.11 ± 0.09 in AS (p<0.05).

In conclusion, both acute and chronic Iso treatment in vivo altered cardiac contractility and CaMKII activity and/or expression. Future work will explore possible correlations to determine whether drugs that exhibit cardiotoxic or therapeutic effects in vivo may work via actions on CaMKIIδ, thus identifying CaMKIIδ as a useful marker of cardiac safety.

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Acute elevations in extracellular glucose confer a mild cardioprotective effect to isolated ventricular myocytes

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Hyperglycaemia in patients suffering an acute myocardial infarction is a marker of poor prognosis. This worsened outcome is independent of the patient being diagnosed as diabetic intimating that the hyperglycaemia rather than diabetes per se is the cause of the poor prognosis. Paradoxically, infarct size is often smaller in diabetic subjects compared to non-diabetics suggesting some form of cardioprotection may exist. To investigate whether glucose itself imparted a cardioprotection to ventricular myocytes, cells were isolated from adult male Wistar rats (animals were humanely sacrificed in accordance with Home Office regulations). Isolated cells were then subjected to a chemically mimicked ischaemia/reperfusion injury (I/R) model. Briefly, cells were paced at 1 Hz with electric field stimulation at 32°C and perfused with normal Tyrode (NT) solution for 2 minutes, followed by 7 minutes with substrate-free metabolic inhibition Tyrode (SFT) containing 2 mM cyanide and 1 mM iodoacetic acid. Cells were then ‘reperfused’ with NT for 10 minutes and the cardio-
Arterial stent intimal hyperplasia: role of hypoxia and blood-wall oxygen transport

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Intimal hyperplasia (IH) causes failure of interventions, but its causation is unclear. Low wall shear is implicated at bypass grafts and wall damage at stents. In addition, stenting deform arteries, inducing wall hypoxia ¹, ² but that occurrence has attracted limited attention.

Our interest in stent-associated IH arose because: oxygen transport between luminal blood and arterial wall is fluid-phase controlled; arterial sites which experience low wall shear may be hypoxic; and arterial curvature and branching are commonly non-planar, generating swirling and cross-mixing which can increase wall shear and blood-wall mass transport, including of oxygen ³.

In previously published work we developed a stent with a helical centreline, implanting it (US Laboratory Animal Welfare Act) in one common carotid artery (CCA) of 10 healthy pigs, and a conventional (straight) stent contralaterally. The former stent deformed vessels helically, causing swirling and cross-mixing of flow. At sacrifice, one month after implantation, transverse sections showed significantly less intimal thickening in helically-stented than straight-stented vessels ⁴.

To increase understanding, we measured in the same sections intimal thickness and medial area, and calculated average intima-media ratio (IMR) and total adventitial area. We also counted adventitial vessels (circular or quasi-circular contours) under low power magnification – average number per section: helical 130; straight 220.

Vessel number was not correlated with section thickness, consistent with the measurement procedure. The results are therefore presented as vessel density per unit adventitial area. IMR was significantly lower in helically-stented than straight-stented arteries (0.75±0.66 vs 1.20±0.35, P<0.01) as was adventitial vessel density (40.1±16.8 vs 61.0±26.6, P<0.01).

Less intimal thickening in helically-stented than straight-stented CCAs is not readily explained by wall damage. Inspection of arterial sections suggested helical stents caused wall thinning at convex helical bends and wall thickening at concave helical bends. Helical stenting can generate several fluid mechanical changes, including luminal cross-mixing, which can increase wall shear stress and convective blood-wall mass transport, including of oxygen.

These effects are not readily distinguished. However, if adventitial vessel density can be considered surrogate for wall hypoxia, it is implied that wall hypoxia was less in helically-stented than straight-stented CCAs, consistent with the importance of conduit geometry and intraluminal mixing, and with wall hypoxia being a significant causative factor for IH. Supportive of that view, supplementary oxygen reduced the severity of IH after arterial stenting in animals ⁵.

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Mechanism of hydrogen sulphide mediated contraction in rat small pulmonary arteries


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The gas H₂S (hereafter ‘sulphide’) may act as an endogenous vasodilator in the systemic circulation. In pulmonary arteries, however, sulphide elicits a biphasic contraction with an unknown mechanism. We assessed the mechanism of this contraction in rat 2nd order pulmonary arteries (PA), using NaHS as a source of sulphide. Techniques used included recording of isometric tension in PA rings mounted in a small vessel myograph and measurement of reactive oxygen species (ROS) levels in segments of PA using the luminescent ROS indicator LO12 (10 μM). NaHS (500 μM) evoked a complex increase in tension, comprising a small contraction followed by a second and larger contraction which gradually relaxed. The contraction, measured at its peak, was inhibited by 95 ± 5% by the mitochondrial complex 3 blocker antimycin (10 μg/ml, n = 6; p <0.05; paired t test) by 64 ± 7% (mean ± SEM) by the anti-oxidant TEMPO (3 mM, n = 3; p <0.05), and by 78 ± 10 % by the RyR blocker dantrolene (50 μM, n = 4; p <0.05). In 5 experiments, low concentrations (10 or 30 μM) of sulphide caused an immediate short-lived increase in [ROS] measured using LO12. At higher concentrations (100, 300 and 1000 μM), this initial response was followed by a second increase in ROS which developed and then decayed to baseline within 5 minutes. The time courses of the biphasic increases in tension and ROS were similar. The increase in ROS was attenuated by rotenone (1 μM, 29 ± 15% inhibition, n = 5; ns), and very markedly suppressed by a combination of rotenone (1 μM) and antimycin (10 μg/ml) (83 ± 6% inhibition, n = 6; p <0.05).

There is evidence that sulphide is oxidised by the mitochondrial membrane flavoprotein sulphide-quinone oxoreductase (SQR) and that this reaction generates electrons which are donated to ubiquinone and are then passed onto complex 3 (Hildebrandt & Grieshaber, 2008). Given the marked block by antimycin of both sulphide-induced tension development and the increase of ROS it evokes, we propose that metabolism of sulphide by SQR is giving rise to a complex 3-mediated increase in ROS through this mechanism, and that this causes a contraction by activating the RyR. In light of the proposal that sulphide levels rise in PA during hypoxia (Olson & Whitfield, 2010), we further speculate that increases in cellular [sulphide] during hypoxia promote its oxidation by SQR and donation of electrons to complex 3, and that this could account for the apparent paradox that hypoxia may be associated with an increased ROS production by complex 3 in pulmonary artery smooth muscle cells (Waypa et al., 2001).

Hildebrandt TM & Grieshaber MK (2008) FEBs Journal275, 3352-3361


The Wellcome Trust: Programme Grant 087776

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Protein kinase C delta isoform shRNA plasmid construct restores vascular disorders in spontaneously hypertensive rats

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It is known that protein kinase C (PKC) family involved in arterial hypertension development due to its overexpression and related decrease in BKCa channels activity. The vascular smooth muscle force development is closely coupled to membrane potential and changes in conductivity for K⁺ ions carried in rat thoracic aorta mainly through BKCa channels which, in turn, appear to be PKC dependent.

The goal of this study was to clarify the PKC role in hypertension development in SHRs and to make an attempt to reduce vascular abnormalities related to ion channelopathy and vascular hypercontractivity in SHRs using siRNA and plasmid-based siRNA.

Experimental design of the study comprised RT-PCR, patch-clamp technique and systolic blood pressure measurement in non-anesthetized rats using calf tail Sphyngomanometer S-2 (Hugo Sachs Elektronik, Germany). Vector system pSilencer-siPKC was constructed and used for suppressing PKC expression in a target-specific manner. siRNA and pSilencer-siPKC were injected intravenously via the tail vein in conscious SHRs. The rats were then killed on the 7th day by cervical dislocation following ketamine (45mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia.

It was found that protein kinase C delta isoform gene silencing using both siRNA and pSilencer-siPKC in SHRs led to significant decrease in arterial blood pressure by 16 +/- 5 mmHg and 17 +/- 4 mmHg, respectively, on the first intravenous post-injection day and persisted for 7 days after. To investigate the effect of post-transcriptional gene silencing on BKCa, the patch-clamp technique was used. It was shown that outward ionic currents was 52 ± 5 pA/pF in healthy rats, and 25 ± 2 pA/pF in SHRs. In SHRs treated with siPKC, siRNA BKCa current density was 35 ± 3 pA/pF, and in SHRs treated with pSilencer-siPKC it was 36 ± 3 pA/pF (P<0.05, n=24).

In conclusion, the silencing of PKC gene expression using both siRNA and plasmid-based siRNA led to an increment in BKCa channels activity and arterial blood pressure normalization.

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Inhibition of HDAC8 and acetylation of Hsp20 regulates contractile activity of human myometrial smooth muscle

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Recent evidence has implicated a role for Histone deacetylases 8 (HDAC8) as a cytosolic marker of smooth muscle cells and
an important regulator of contractility in vascular smooth muscle cells (1). There is now rising evidence to indicate that acetylation of non-nuclear proteins is an important post-translational modification regulating many cellular functions including the cytoskeletal architecture of cells and in smooth muscle physiology, these include proteins involved in the contractile apparatus (2). The aim of this study was to examine if inhibition of HDAC8 will lead to relaxation of myometrial smooth muscle and to find the putative protein targets of its enzymatic activity.

The effect of the specific HDAC8 inhibitor, Compound2 (Comp2, 100μM in DMSO) (3) was tested on contractions in isometrically mounted myometrial strips from biopsies obtained from pregnant women following Cesarean section. Integrals from selected 30 min intervals prior to and post treatment/control were calculated and compared between groups using paired t test. Treatment with Comp2 significantly inhibited (p<0.05) spontaneous (98±2.41 vs. 71±6.9) and oxytocin-induced (94±3.1 vs. 71±4.3) contractions after 20 min lasting up to 1.5h. Importantly, this occurred without elevation of nuclear-resident histone acetylation or marked changes in global gene expression as measured by gene chip arrays (significant change in only 3 out of 47,000 transcripts at 2-fold change, p<0.05, Anova).

Co-immunoprecipitation (co-IP) and immunocytochemistry demonstrated that HDAC8 interacts with α-smooth muscle actin and myosin heavy chain (western blotting, n=3), indicating that they can be potential sites of HDAC8 enzymatic activity. However, co-IPs with antibodies for acetylated Lysine residues and lysates from non-pregnant myometrial tissues treated with Comp2 and a pan-HDACs inhibitor Trichostatin A (TSA, 3.3μM) for 24h did not detect acetylation of these proteins. The same results were obtained for known targets of acetylation: calmodulin, Hsp27, 14-3-3 and cofilin. Heat shock protein 20 (Hsp20) was the only protein in which acetylation via inhibition of HDAC8 was observed. An increase in Hsp20 acetylation correlated with decrease in phosphorylation levels of cofilin after 1h and with significant effects after 5h of treatment (140±45 vs. 36±11, C vs Comp2, p<0.05, Anova, post-hoc Kruskal-Wallis test). This indicates a potential molecular mechanism by which Hsp20 acetylation can affect myometrial activity by liberating cofilin and destabilizing actin filaments (4).

Our studies show for the first time that inhibition of the non-nuclear lysine deacetylase HDAC8 (or KDAC8) regulates the contractile capacity of human myometrial smooth muscle. Walrregny D. et al., 2005 Faseb J., 19:966-968.


This work was funded by MRC grant G0800202

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Isolated perfused pig bladder: a novel experimental approach for the study of whole organ physiology in large animals

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Our knowledge of bladder function remains incomplete and experimental approaches such as muscle strip experiments, though central to our current understanding, fail to reproduce the complex network of cell-cell interactions that underpin normal bladder function. Improving our understanding of bladder physiology necessitates the use of whole organ preparations and to date most of this work has been done using bladders from small rodents. Extrapolating findings generated using small laboratory animals to lower urinary tract physiology of large animals and to the clinical context is difficult, and requires careful validation. In order to overcome some of these limitations, the aim of this study was to develop an experimental model using an isolated whole pig bladder, which could be used to study the integrative physiology of the bladder.

Pig bladders and their associated vasculature were retrieved from the abattoir, with a mean warm ischaemia time of 30 mins and a mean cold ischaemia time of up to 120±15 mins. A temperature-controlled organ bath with an integrated arterial pump-perfusion system was designed to preserve organ viability. The pig bladders were perfused with Krebs-bicarbonate buffer for a period of several hours and intravesical and intra-arterial pressures were monitored. Validation of tissue viability involved assessment of histology, arterial perfusion and pH. Cholinergic stimulation was undertaken by administration of carbachol (1μM-3mM) through either the vascular or intravesical route.

Once perfused, the bladders remained viable on structural, perfusion and biochemical testing for a period of over 6 hours. Histologically, light microscopic examination of haematoxylin and eosin stained bladder biopsies taken after 6 hours of perfusion demonstrated well-preserved tissue architecture with no evidence of autolysis or necrosis. There was also no evidence of tissue ischaemia as no significant changes were recorded in the pH of the perfusate before and after its passage through the organ. Intravascular administration of carbachol elicited contractions of the pre-filled bladder (N=5) in a dose dependant manner with a maximum response of 98.8 ± 1.2 cmH2O and an EC50 of 96.7 ± 22.7 μM. Intravesical carbachol (300μM-3Mm) also elicited bladder contraction. Using a pump perfusion system, the viability of an isolated large animal bladder can be maintained for a prolonged period of time even after a limited period of warm and cold ischaemia. This experimental approach can be used to study whole organ function of large animal bladders, and the potential for different routes of drug administration may help discern the relative contribution of different cell types in tissue function.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Evidence of a role for astrocyte purinergic signalling in regulation of extracellular potassium and action potential propagation in central nervous system white matter

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It is now recognized that astrocytes in the central nervous system (CNS) communicate with each other by an ATP-mediated rise in intracellular calcium ([Ca2+]i), called Ca2+ signaling. Astroglial Ca2+ signaling has been studied extensively in grey matter synaptic regions, but its functional significance remains unclear. A key function of astrocytes is considered to be uptake of K+ released by axons during action potential propagation, which is essential for sustained neuronal activity. Therefore, we examined whether glial ATP-mediated communication has a role in K+ regulation and maintenance of axonal excitability in the rat optic nerve, a CNS white matter tract. Wistar rats aged postnatal day (P) 15-30 were killed humanely in accordance with the UK Animals (Scientific Procedures) Act (1986), and optic nerves were isolated and maintained in a brain slice chamber continuously perfused with artificial cerebrospinal fluid (aCSF). Changes in glial [Ca2+]i, nerve compound action potential (CAP) and [K+]o were measured in the presence of a range of purinergic receptor (P2R) modulators, for both ionotropic P2XR and metabotropic P2YR: (100μM unless otherwise stated) general P2XR/P2YR antagonist suramin; P2XR antagonist PPADS; P2X7R antagonist oATP; P2X7R agonist BzATP (10μM); P2Y1 agonist MRS2179; thapsigargin (10μM) to block Ca2+ release from intracellular stores. Calcium imaging in fura-2 loaded nerves indicated that ATP evoked raised glial [Ca2+]i predominantly via P2Y1R and P2X7R, and was significantly decreased by suramin, MRS2179, PPADS, oATP and thapsigargin. In addition, stimulation of axonal action potentials at 35Hz evoked a rise in glial [Ca2+]i, that was significantly reduced by suramin (p<0.05, t-tests, n=5). Electrophysiological measurement of [K+]o (using K+-selective micro-electrodes) and the CAP showed that increasing the stimulation frequency to 35Hz for 120s results in an activity-dependent increase in [K+]o, and decay in nerve conduction, followed by a post-stimulus clearance of K+ and nerve hypoeexcitability, and finally K+ redistribution and recovery of nerve conduction. Treatment with the P2 purinergic receptor antagonists suramin, PPADS, MRS2179, oATP, or with thapsigargin or zero [Ca2+]i, resulted in a significantly greater activity-dependent decay in the CAP compared to aCSF controls (p<0.01, t-tests, n=5). We tested the effects of suramin, oATP and thapsigargin on [K+]o during 35Hz stimulation and all resulted in a significantly greater rise in [K+]o compared to controls (p<0.05, paired t-tests, n=5). The results show K+ regulation and nerve conduction are reduced when P2R are blocked. The study provides evidence that ATP-mediated astrocyte Ca2+ signaling is important for K+ regulation and maintaining axonal activity in CNS white matter.

Supported by the MRC.

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Oral Communications

C86

Human embryonic stem cell derived functional astrocytes are neuroprotective through glutathione dependent and independent mechanisms

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Neurodegenerative diseases represent a major healthcare burden, are incurable and without effective treatments. While rodent research has demonstrated the importance of glial-neuronal interaction, inter-species differences limit translation. Human embryonic stem cells (HESC) offer an opportunity to model human injury in vitro and allow clinical extrapolation. Experiments were performed across two independent HESC lines (H9/Hues9); values are mean ± SEM; n=3; p<0.05 was held significant; data were analysed by t test and ANOVA with Newman-Keuls post-hoc analysis; Cell viability was assayed by CellTiter-Glo (Promega) and confirmed by TuJ1/activated PLC.

Human embryonic stem cells (HESC) were cultured and neuralised by establishment of neurons (TuJ1: 95.5 ± 1.4%). Highly enriched astrocyte populations were derived from differentiation of late NPCs in BMP4/LIF (GFAP: 95.7 ± 3.1%; $100b: 90.1 ± 2.0%). Their functional nature was verified by H3-glutamate uptake. Enriched populations of HESC derived neurons (TuJ1: 95.5 ± 1.4%), were challenged with 50μM H2O2, which caused 42.2 ± 1.4% cell death. Treatment of HESC neurons with astrocyte conditioned medium (ACM) reduced cell death (31.0 ± 1.8% p<0.05), which was unchanged upon astrocyte pretreatment with buthionine sulfoximine (BSO), a glutathione dependent mechanism. These data suggest that ACM is protective by glutathione independent mechanisms, such as GDNF production (2). There is growing evidence for synthetic triterpenoids as potential therapeutic agents in mouse models of neurodegenerative disorders (3). In order to extend these observations to a human system, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-TFEA), an enhanced(3) shortened glial cell line-derived neurotrophic factor (GDNF), was employed to test the neuroprotective ability above untreated ACM (19.6 ± 1.7% p<0.05, amenable to BSO mediated inhibition (31.6 ± 1.4%). Therefore the additive protective effect of CDDO-TFEA is mediated indirectly by the astrocyte by a glutathione dependent mechanism. These data provide important proof of concept that HESC derived neurons and astrocytes can be used to model neurological injury and protection, and in addition provide a key platform for studies emerging using human iPScells. Joannides et al. (2007). Stem Cells 25, 731-7

Hampton et al. (2010). J Neurosci 30, 9973-83


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C87

Are astroglial Gq-protein coupled receptors functionally relevant?

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It is widely accepted that astrocytes release gliotransmitters to modulate synaptic transmission and that this release is at least partially Ca2+-dependent. However, recent publications (1,2) have reported that in two mouse models, MrgA1* and IP3R2 knockout, Gq-protein coupled receptor (Gq-PCR) mediated Ca2+ signalling in astrocytes does not affect synaptic transmission or short- and long-term plasticity. These observations questioned the importance of signalling via Gq-PCR activated pathways in astrocytes. We have used optogenetics to determine if Gq-PCRs on astrocytes are functionally relevant.

An adenoviral vector (Ad.BS.GfaABC1D.Opto1_skip.Gal4P65) was generated which employs an enhanced(3) shortened glial fibrillary acidic protein promoter to express an opsin-GPCR chimera - Opto1AR, adrenergic receptor (Opto1AR) specifically in astrocytes. To verify that this construct activates phospholipase C (PLC), cultured primary rat astrocytes were transduced with Ad.BS.GfaABC1D.Opto1AR, skip.Gal4P65. 48 hours later, they were loaded with the Ca2+ indicator Rhod-2AM, super-fused with HBSS (34°C) and imaged using Leica SP1 confocal microscope. Stimulation with 470nm light, induced rapid increases in [Ca2+]i (fluorescence increase +187.2% ± 13.91, n=36 cells from 6 cover-slips). The PLC inhibitor U73122 (10μM) blocked light-induced [Ca2+]i increases in Opto1AR-transduced astrocytes (-2.51 ± 0.8%, n=10 cells from 6 cover-slips, p<0.001). These data confirm that Opto1AR signals through PLC.

Next, we investigated if activation of Opto1AR can induce responses in nearby neurones. We focused on the locus coeruleus (LC), the main noradrenergic (NAergic) nucleus innervating the forebrain. We engineered a viral vector (Ad.Superi.PRSx8.TN-XXL) to drive expression of a Ca2+ indicator, TN-XXL (4) in NAergic LC neurones. Organotypic rat brainstem slices containing LC were prepared using methods described previously (5) and transduced with Ad.Superi.PRSx8.TN-XXL and Ad.BS.GfaABC1D.Opto1AR, skip.Gal4P65. Stimulation of astrocytes with 445 nm light induced rapid increases in the YFP/CFP ratio in TN-XXL-expressing LC neurones (+58% ± 3.5, n=25 cells in 10 slices, p<0.01), indicating that they were activated by light-stimulated astrocytes. In vivo, optogenetic activation of Opto1AR expressed in the rostral ventrolateral medulla, an area containing catecholaminergic neurones involved in blood pressure control, triggered robust cardiovascular and sympathetic responses in α-chloralose anaesthetised rats (100mg/kg i.V, supplemented with 20 mg/kg as required). These experiments indicate that signalling mediated by Gq-PCRs in astrocytes is physiologically relevant and may be important in central autonomic control.


Supported by the BBSRC, British Heart Foundation and Wellcome Trust.

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C88

Differential control of striatal inhibition by histamine
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The striatum receives input from the histaminergic system arising in the hypothalamus. Histaminergic neurons exhibit a diurnal rhythm in their activity, being active during wakefulness and relatively silent during sleep. Consequently, the striatum is likely to experience a relatively high concentration of histamine during wakefulness. This, combined with the high expression of histamine receptors in the striatum, suggests that histamine might have a regulatory role in striatal function. We examined the effect of histamine on the main excitatory and inhibitory inputs to the principal neuron of the striatum, the GABAergic medium spiny projection neurons (MSNs). We performed whole-cell patch-clamp recordings of single MSNs, pairs of connected MSNs and pairs of connected interneurons and MSNs in acute slices of mice. We investigated the effect of bath-applied histamine (10 μM) in conjunction with selective histamine receptor antagonists on the excitatory responses evoked by cortical or thalamic stimulation or inhibitory responses evoked by local stimulation or unitary inhibitory responses between pairs of neurons. Both cortical and thalamic inputs to MSNs were negatively modulated by bath applied histamine, which was prevented by co-application of the H3 receptor antagonist, thioperamide (10 μM). Similarly, inhibitory inputs evoked by local stimulation were negatively modulated by bath applied histamine. However, in paired recordings we found that only the unitary inhibitory responses between pairs of MSNs were significantly reduced by histamine, whereas those between fast spiking (FS) interneurons and MSNs were unaffected. Histaminergic innervation of the striatum is thus involved in the negative regulation of both of the main excitatory inputs and the feed-back inhibitory input to MSNs, but does not affect the feed-forward inhibitory input from the FS interneurons. The selective attenuation of feed-back inhibition by histamine will alter the dendritic processing of excitatory inputs and thus the expression of basal ganglia function.

This work was supported by the MRC and the European Community.

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C89

Investigating glycine-activated currents in rat substantia nigra neurones
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We have previously shown that dopaminergic and non-dopaminergic neurones in the early postnatal rat substantia nigra (SN) are labelled by antibodies raised against NR3A and NR3B NMDA receptor subunits. NR3 subunits, unlike NR2 subunits, form diheteromeric NMDA receptors that are gated solely by glycine rather than by glutamate and glycine. Glycine-activated NR1/NR3 receptors are non-selective cation channels which are excitatory, by contrast with the well-established inhibitory role of glycine-gated Cl- channel receptors. In order to determine whether SN neurones express functional glycine-activated NR1/NR3 NMDA receptors we have made whole-cell patch-clamp recordings from SN neurones in midbrain slices prepared from rats aged 5-16 days old (decapitated under halothane anaesthesia). SN neurones were voltage-clamped to -60 mV using a patch pipette filled with either a high [Cl-] solution to set ECl at 0 mV, or a low [Cl-] solution to set ECl at -55 mV. Glycine (1 mM) was applied from a picospritzer (10 p.s.i. for 1s) in the presence of a cocktail of inhibitors in the perfusion solution and in the picospritzer pipette (strychnine, 600 nM; D-AP5, 50 μM; picrotoxin, 50 μM; tetrodotoxin, 100 nM) to block glycine-gated Cl- channels, NR2-containing NMDA receptors, GABA_A receptors and action potential-dependent neurotransmitter release respectively.

When ECl was set to 0 mV, glycine evoked an inward current of ~2095 ± 697 pA (n = 4). When ECl was set to -55 mV to minimise Cl- conductances, a glycine-evoked inward current was still observed at -60 mV although this was smaller in amplitude (-387 ± 59 pA; n = 7). Glycine-activated outward currents were seen at +40 mV (2285 ± 355 pA; n = 4). Repeated applications of glycine (every 200s for 1200s) caused a decrease in the amplitude of glycine-activated currents ([Glycine(1200s)/Glycine(200s)] ratio, 0.19). These data are consistent with a glycine-activated excitatory cation current, suggesting that functional NR1/NR3 NMDA receptors may be present in developing SN neurones.

Supported by a BBSRC PhD studentship to JH

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Electrophysiological deficits at GABAergic synapses on Purkinje cells of the dystrophin-deficient mdx mouse: possible implications for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) results from a absence of the protein dystrophin. It is characterized by severe wasting of skeletal muscle. There is also a significant, but often overlooked, accompanying cognitive and behavioural deficit(1). In the cerebellum dystrophy is normally localized at the postsynaptic membrane of GABAergic synapses located at the synapse in cerebellar Purkinje cells of the dystrophin-deficient mdx mouse. Whole-cell patch-clamp recordings of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were performed in cerebellar slices from mdx and littermate control mice. Using non-stationary noise analysis, we found a significant difference in the number of functional GABAergic channels located at GABAergic synapses in mdx mice (38.38 ± 2.95; n=14) compared to littermate controls (53.03 ± 4.11; n=12) (p = 0.01).

In response to the application of the GABA agonist gaboxadol we found a significant difference in the number of receptors at GABAergic synapses in mdx mice (37.36 ± 3.82 pA; n=8) compared to littermate controls (65.01 ± 5.89 pA; n=9). These results suggest that in cerebellar Purkinje cells of dystrophin-deficient mdx mice there is a reduction in the number of receptors localised at GABAergic synapses, and an accompanying increase in extrasynaptic GABAergic receptors, indicating that dystrophin plays an important role in ion channel localization and stabilization at the post-synaptic membrane. If similar changes occur in the CNS in boys with DMD, it may impact on the function of neural networks and contribute to motor, behavioural and cognitive impairment apparent in many boys with DMD.


This work was supported by a grant from the Muscular Dystrophy Association, USA (3964)

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TRPM7 cation channel is overexpressed in human pancreatic ductal adenocarcinoma and is required for cancer cell migration

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a poor prognosis: mortality essentially equals incidence. Despite many advances in medicine, there is still a lack of early diagnostic biomarkers of PDAC as well as efficient therapeutic strategies in advanced cases. Indeed, surgery is currently the only hope for cure. Thus, a better understanding of the cellular and molecular mechanisms involved in the development and progression of PDAC is needed. The melastatin-related transient receptor potential 7 channel (TRPM7) is a non-selective cation channel which has been shown to regulate the cell proliferation in human head and neck (Jiang et al., 2007), breast (Guilbert et al., 2009) cancers and PDAC (Yee et al., 2010) as well as migration in nasopharyngeal cancer (Chen et al., 2010). However, the role of TRPM7 in PDAC progression is far from understood. TRPM7 expression was studied using quantitative RT-PCR and immunohistochemistry in human PDAC tissues in comparison with pancreatic exocrine tissues from healthy donors. TRPM7 activity was studied in BxPC3 cell line by the whole-cell patch-clamp technique, and calcium/magnesium-imaging. The contribution of TRPM7 to cations entry was assessed using the Mn2+-quenching technique. Finally, TRPM7 physiopathological role was studied using MTT assays and cell migration tests in Boyden chambers. Our results showed that TRPM7 is overexpressed 13-fold in cancer tissues compared to the normal ones. Furthermore, TRPM7 staining was stronger in poorly differentiated PDAC samples than in well and moderately differentiated ones, suggesting a correlation between TRPM7 expression and PDAC progression. In BxPC3 cell line, dialyzing the cytoplasm during the whole-cell recording with a 0-Mg2+ solution activated a non-selective current with a strong outward rectification. This cation current was inhibited by intracellular Mg2+ with a maximal inhibition for free-[Mg2+]i=850 μM. This magnesium inhibited cation current was dramatically inhibited by a siRNA targeting TRPM7 (siTRPM7) compared to scrambled siRNA. Mn2+-quenching of Fura-2 was decreased by siTRPM7 suggesting that TRPM7 contributes to divalent cations entry in BxPC3 cells. Indeed, siTRPM7 induced a decrease of basal intracellular Mg2+ fluorescence ratio (F360nm/F380nm) but not of intracellular Ca2+. Finally, siTRPM7 decreased BxPC3 cell migration by 55% without affecting the cell proliferation. Cell migration was fully restored by adding 1mM Mg2+ in culture media after siTRPM7 treatment.

In conclusion, TRPM7 is overexpressed in PDAC and regulates pancreatic cell migration likely via a Mg2+-dependent mechanism. Since the TRPM7 expression is stronger in undifferentiated areas, we propose this channel as a potential biomarker of poor prognosis in PDAC.


The effect of inflammation on human airway epithelial glucose transport and GLUT transporter expression

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The glucose concentration of the airway surface liquid (ASL) is tightly regulated by the airway epithelium, which is vital to prevent airway infections. This involves, in part, glucose uptake into airway epithelial cells through facilitated glucose transporters GLUTs (Kalsi et al, 2008). Airway inflammation could disrupt glucose homeostasis in airway epithelial cells via this mechanism. The aim of this study was to investigate the effects of inflammation induced by pro-inflammatory cytokines on glucose transport and expression of GLUT transporters in human H441 and primary human bronchial epithelial cell (HBEC) cultures. Cells were grown at air liquid interface for 7-14 days to form confluent polarised monolayers. Inflammation was induced with a cocktail of cytokines [Tumor Necrosis Factor α (100 ng/ml), Interferon γ (100 ng/ml), Lipopolysaccharide (10 μg/ml)] for 0, 24, 48 and 72 hours. Transepithelial electrical resistance (TER) of the monolayers was measured using an epithelial voltohmmeter. The effect of cytokine treatment on glucose uptake was assessed using radiolabelled glucose (14C-D-glucose, 1 μCi) applied to the apical or basolateral surface. GLUT-specific transport was assessed using the pharmacological inhibitor, phloretin (1 mM). Cytokine-induced inflammation significantly reduced H441 and HBEC monolayer TER in a stepwise manner over 72 hours, from 627 ± 40 to 278 ± 15 Ω.cm² (P<0.0001; n=8) and 440 ± 79 to 221 ± 56 Ω.cm² (P<0.05; n=4), respectively. This drop in TER inversely correlated with an enhanced glucose uptake in both H441 and HBEC monolayers. Apical glucose uptake in HBEC monolayers increased by 265 ± 53% (P<0.05; n=3) after 72 hours of cytokine exposure. Comparable apical glucose uptakes were observed in H441 monolayers. No significant difference in basolateral glucose uptake was seen in either cell type. Similarly, when studying GLUT-specific uptake in H441 monolayers, an increase in phloretin-sensitive glucose uptake was present across the apical membrane, but not the basolateral. Western blotting analysis and fluorescence immunocytochemistry (IHC) showed the presence of GLUT2 expression in both HBEC and H441 cells (GLUT2 blocking peptide was applied as a control). Interestingly, upon cytokine exposure, GLUT2 expression noticeably increased over 72 hours in H441 monolayers when observed by IHC. In conclusion, inflammation induced by cytokine exposure had a significant affect on epithelial resistance and apical glucose uptake in both HBEC and H441 cell monolayers. The increase in GLUT-specific glucose uptake corresponds with enhanced GLUT2 expression, suggesting GLUT2 may play an important role in regulating ASL glucose concentration under inflammatory conditions. Kalsi KK et al. (2008). Eur J Physiol 456, 991-1003.

Supported by the Wellcome Trust 088304/Z/09/Z

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Non-Genomic Estrogen Regulation of Airway Surface Liquid Height in Normal and Cystic Fibrosis Bronchial Epithelia

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Male cystic fibrosis (CF) patients survive 9 years longer than females (CF gender gap) and lung exacerbations in CF females vary during the estrous cycle. Estrogen has been reported to reduce the height of the airway surface layer (ASL) in female CF bronchial epithelium and we have previously shown an anti-secretory effect of estrogen in epithelia. Here we investigated the role of estrogen receptors and basolateral K⁺ channels in mediating estrogen effects on ASL height in normal (NuLi-1) and F508del CF (CuFi-1) bronchial epithelium monolayers grown on Transwell filters in an air-liquid interface. Confocal fluorescence microscopy experiments revealed that ASL height was significantly higher in the normal bronchial epithelial NuLi-1 cell line compared to the F508del CuFi-1 cells (NuLi 6.82 ± 0.33 μm vs CuFi 5.58 ± 0.14 μm, n=20, p<0.001). 17β-estradiol (E2, 0.1 to 10nM) reduced ASL height in both normal (25% decrease, n=5, p<0.05, ANOVA) and CF (20% decrease, n=5, p<0.05, ANOVA) cell lines after 30 min treatment. Treatment with the Cl⁻ transport inhibitor bumetanide (10 μM) or the KCNQ1 K⁺ channel inhibitor chromanol HMR1556 (1 μM) decreased ASL height significantly in both NuLi-1 and CuFi-1 epithelia. E2 had no additive effect on ASL height in the presence of these ion transporter inhibitors. Moreover E2 decreased bumetanide-sensitive transepithelial Cl⁻ current in normal cells (E2: 6.47 ± 2.08 μA/cm² vs control: 9.52 ± 2.08 μA/cm², n=3, p<0.05, paired t-test) and produced a small increase in amiloride (10μM) sensitive current in CF cells (E2: 8.75 ± 1.39 μA/cm² vs control: 7.14 ± 1.38 μA/cm², n=5, p<0.07, paired t-test). Treatment with the nuclear-impeded Estrogen Dendrimer Conjugate (EDC 1nM E2 equivalent concentration) produced a significant reduction in ASL height in both cell lines (4.72 ± 0.33 μm in NuLi-1 and 4.86 ± 0.42 μm in CuFi-1, n=5, p<0.05, ANOVA) whereas the empty dendrimer had no effect. These results demonstrate that estrogen decreases Cl⁻ secretion, K⁺ recycling and ASL height in both CF and normal bronchial epithelial cells. These rapid responses to E2 are membrane-initiated rather than via the classical nuclear receptor signal transduction pathway and target the Cl⁻ secretory pathway and basolateral KCNQ1 K⁺ channels. The ion transporter inhibitor data indicate that E2 acts on ASL by inhibiting chloride secretion in normal cells and increasing sodium absorption in CF cells.

This work was supported by a NIHP Ireland Career Enhancement and Mobility Fellowship to V.S.C. co-funded by EU FP7 Marie Curie Actions and the Irish Higher Education Authority. Support from the Higher Education Authority of Ireland PRTLI Cycle 4 (to B.J.H) and the National Institutes of Health (R37 DK015556, to J.A.K.) is acknowledged.

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Genetic inactivation of the KCNN4 K⁺ channel protects from lethality in a cystic fibrosis mouse model
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Cystic fibrosis (CF) is produced by mutations in the CFTR Cl⁻ channel gene that dramatically reduce epithelial electrolytes and fluid secretion and maintenance of a hydrated mucosa. Mouse models are useful to study the intestinal complications of CF. Electrogenic Cl⁻ secretion is strictly coupled to the activity of basolateral cAMP- and Ca²⁺-dependent K⁺ conductances. We have demonstrated that KCNN4 corresponds to the Ca²⁺-activated K⁺ conductance in the basolateral side of intestinal epithelium and that, its genetic silencing in mice abolishes Ca²⁺-activated Cl⁻ secretion and reduces water content of the stools (Flores et al., 2007). The Ca²⁺-dependent Cl⁻ secretion in the intestine of CF mice has been postulated as an important factor that increases survival of these animals. Given the role of KCNN4 in regulating secretion we investigated if the abolition of KCNN4 activity in a mouse model of CF can modify the intestinal phenotype of the disease.

Control (WT), KCNN4 KO and mutant CFTR-KO mice were used. Breeding of double mutant animals was done by mating of double heterozygous. Survival and weight progression curves for the offspring were constructed. After killing, dissection of the intestine was performed to obtain tissue samples for periodic Schiff Acid (PAS) staining. Electrogenic secretion of the colon was studied by Ussing chamber technique. Water content of the faeces was also measured.

Genetic silencing of KCNN4 reduced the lethality of CF mice from 43% to 3% after 60 days (13 and 3 dead out of 30 mice on each group respectively). The mean body weight of CF animals at 40 days of age was 10.8 ± 0.8 g, almost half of the weight recorded in WT and KCNN4 KO animals (19.8 ± 0.8 and 19.2 ± 1.2 g respectively). The double mutant did not show any improvement in weight (10.7 ± 0.7 g) compared to CF. Electrogenic cAMP- and calcium-activated currents were absent in colon from both CF and double mutant animals. Water content of stools was reduced in the KCNN4 KO (46 ± 1.6 %) with respect to WT (57 ± 0.8 %), CF (51 ± 2 %) and double mutant (53 ± 2 %) animals. PAS staining demonstrated that both CF and double mutant animals had and increased accumulation of mucus in the intestinal crypts.

Our results show that genetic silencing of KCNN4 channels greatly improved the survival of animals bearing mutant CFTR channels. The observed reduction in lethality does not seem to be related to improvement of the intestinal transport function since double mutant mice did not show alternative chloride conductances, changes in water content in faeces, reduced accumulation of thick mucus or increased body weight when compared to CF animals. Since KCNN4 is broadly expressed in mammalian tissues we now aim at studying the role of KCNN4 in non epithelial cells that could be pathologically affected in the CF.


Supported by FONDECYT 11100408 and Conicyt PFB.

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Loss of DRA expression results in severely decreased murine colonic HCO3⁻ secretion, low surface pH, disturbed mucus barrier, signs of colonic mucosal inflammation, and increased susceptibility to DSS-induced injury
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Background: DRA (downregulated in adenoma, Scl26a3) is a member of the SLC26 family of anion transporters which is mutated in congenital chloride diarrhea (CLD) (1). Its genetic ablation interferes with duodenal mucosal HCO3⁻ secretion and increased stool Cl⁻ and water content (2,3). However, CLD patients also have a higher than average incidence of intestinal inflammation (4,5). Aim of the study: In order to search for potential explanations for this clinical finding, we measured colonic HCO3⁻ secretion, epithelial surface pH, and mucus release in vivo and in vitro, searched for molecular and histological signs for mucosal inflammation, and investigated the susceptibility for DSS-induced intestinal inflammation, in DRA-deficient mice and WT littermates. Methods and Results: HCO3⁻ secretory rate (HCO3⁻), measured by single-pass perfusion in vivo and in isolated mid colonic mucosa in Ussing chambers in vitro, as well as epithelial surface pH, measured by two-photon microscopy in exteriorized mid colon of 1.4% isoflurane-anesthetized DRA-deficient mice in vivo, was significantly reduced. The speed of buildup of a fresh mucus layer, measured by two photon microscopy in vivo, was not different to controls. However, no firm adherent mucus layer was present, implicating that neutral pH may be necessary for optimal mucus gel formation. Prolinflammatory cytokine expression was significantly elevated in DRA⁻/⁻ colonic mucosa, which also displayed signs of increased neutrophil infiltration. In addition, DRA⁻/⁻ mice were more susceptible to the development of DSS colitis. Conclusions: Knockout of DRA results in a severely reduced colonic HCO3⁻ secretory rate, a low epithelial surface pH, a lack of firmly adherent mucus, but a normal speed of overall mucin layer buildup in the colon. The mice also displayed signs of colonic mucosal inflammation and a severely reduced resistance to the development of DSS colitis. The data suggest a relationship between colonic HCO3⁻ secretion, mucus layer stability, mucosal protection, and the development of intestinal inflammation.


Renal Cyst Fluid From Human Polycystic Kidney Disease Patients Stimulates Cl− Secretion: Characterization of the Active Factor and Target Channels

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the growth of epithelial lined, fluid filled cysts predominately in kidney tubules and liver bile ducts. Despite the formation of multiple, slowly growing cysts, renal function is usually not severely compromised until the 5th decade of life but then declines rather precipitously (1). Renal injury exacerbates cyst formation and expansion (2). Cyst expansion is thought to result from stimulation of ion secretory events that cause increased movement of electrolytes and, secondarily, water into the cyst lumen. In ADPKD renal tissue, several groups have observed increased intracellular Ca2+ that stimulates the cyst fibrosis transmembrane conductance regulator (CFTR) in the apical membrane of epithelial cells lining the cysts. Interestingly, cyst fluid obtained from human patients can also stimulate secretory activity in renal epithelial cells.

We used electrophysiological techniques to examine the effect of cyst fluid on the mpkCCDcl4 (mouse principal cells of kidney cortical collecting duct, clone 4) cell line. A 10% (V/V) addition of cyst fluid to the basolateral media stimulated two different Cl− channels, a GlyH101-inhibitable CFTR and a tannic acid inhibitor activatable Cl− channel. We identified the active secretory component of the cyst fluid as lysophosphatic acid (LPA). A dose response for LPA showed that concentrations above 0.05 μM stimulated maximal secretory responses in mpkCCDcl4 Cells. Tandem mass spectrometry was used to measure the concentration of LPA in the cyst fluid collected from one patient. The cyst fluid contained 4.0 ± 0.4 μM 16:0 LPA and 1.4 ± 0.04 μM 18:1 LPA (mean ± SEM, quadruplicate), an amount sufficient to stimulate a maximal response. Pretreatment with N-palmitoyl-L-serine phosphoric acid, an LPA receptor antagonist, completely inhibited the secretory response to cyst fluid (n = 4). Pretreatment with 20 μM dicylgycerol pyrophosphate, an LPA1/LPA3 receptor antagonist substantially inhibited (72.8 ± 4.5%; n=5) the Cl− secretory response. Molecular size separation of the cyst fluid indicated that the LPA activity is confined to a fraction containing molecular weight components larger than 100 kDa, suggesting that the LPA is bound to proteins.

In conclusion, we hypothesize that LPA at concentrations found in cyst fluid or blood will exacerbate cyst growth. Under normal conditions, LPA is bound to proteins in the cyst fluid or blood and, therefore, unavailable for binding to the LPA receptors on the basolateral membrane of cystic epithelial cells. Under conditions such as renal injury or loss of cyst wall integrity due to ageing, the LPA-protein complexes would be released into the interstitial space where they would interact with specific receptors to exacerbate cyst growth.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
increased oxidative stress as measured by TBARS concentrations suggesting a placental compensatory response to raised maternal BMI. A follow up study of the offspring is currently being undertaken up to 18 months of age to establish whether obesity in pregnancy is accompanied with longer term responses.


Project funded by ABBOTT Laboratories, Nottingham Respiratory Biomedical Research Unit and the Nottingham University Hospitals Charity.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C98

Glycaemic and insulin sensitivity during the menstrual cycle in women

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There is conflicting evidence concerning the influence of fluctuations in estradiol and progesterone during the menstrual cycle on glycaemic response and insulin sensitivity (Bingley et al. 2008; Yeung et al. 2010). One reason for this could be the failure to establish accurate menstrual phase definition by analysis of hormone concentrations. The aim of this study was to investigate glucose response to a 75g glucose load and analysis of hormone concentrations (estradiol and progesterone) differed significantly during one complete menstrual cycle (Table 1); however even with accurate menstrual phase definition there was no significant difference in glucose AUC, insulin AUC or insulin sensitivity between the three test sessions. This suggests that fluctuations in estradiol and progesterone during the menstrual cycle may not influence glucose or insulin response to a glucose load.

Table 1. Hormone concentrations for each test session

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Menstruation</th>
<th>Progesterone</th>
<th>Luteal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>51.1 ± 11.84</td>
<td>336.7 ± 96.6</td>
<td>532.9 ± 27.47</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.48 ± 0.17</td>
<td>0.40 ± 0.12</td>
<td>0.48 ± 0.38</td>
</tr>
<tr>
<td>Glucose AUC (mg/dL)</td>
<td>106 ± 62</td>
<td>191 ± 70</td>
<td>313 ± 108</td>
</tr>
<tr>
<td>Insulin AUC (mU/L)</td>
<td>13.33 ± 1.057</td>
<td>336.8 ± 106</td>
<td>1372.5 ± 154</td>
</tr>
<tr>
<td>Matsuda (Insulin Sensitivity)</td>
<td>8.7 ± 2.3</td>
<td>7.5 ± 2.0</td>
<td>8.5 ± 1.5</td>
</tr>
<tr>
<td>HOMA (Insulin Resistance)</td>
<td>1.3 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>1.5 ± 0.5</td>
</tr>
</tbody>
</table>

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C99

The role of Annexin A1 in the manifestation of sexual dimorphisms in murine cerebral and systemic inflammatory responses to endotoxin

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Sexually dimorphic inflammatory responses have been noticed in numerous diseases. As females of childbearing capability are generally less prone than males to morbidity and mortality resulting from inflammatory disease such as sepsis[1,2], it is thought that oestrogen is partly responsible for these differences[3]. Annexin A1 (AnxA1) is an endogenous anti-inflammatory protein that is partly upregulated by oestrogen[4]; therefore, we investigated the role of oestrogen in modulating the neuro- and systemic protection of AnxA1 in experimental murine sepsis.

Male and female C57BL/6 (WT) and AnxA1-null (C57BL/6 back-ground) mice were used. Endotoxaemia was induced by i.p. lipopolysaccharide injection (LPS; 10 mg/mouse) for 2 h, followed by quantification of leukocyte-endothelial cell interactions (L/ECI) in cerebral and mesenteric microcirculation by intravital microscopy under terminal anaesthesia (ketamine 150 mg/kg and xylazine 7.5 mg/kg, i.p.). Next the effects of oestrogen were assessed by ovariectomy (OVX) under isoflurane inhalation anaesthesia (4% for induction, 1.5-2.5% for maintenance in O2, sufficient to abolish a pedal reflex), followed by 8 d with or without oestrogen replacement prior to LPS treatment and intravital microscopy of the brain and mesentery as before. Data were considered statistically significant when P<0.05 by ANOVA with Bonferroni’s post test and are expressed as mean ± SEM. n=5-6 mice/group. LPS increased L/ECI in both vascular beds in all animals. OVX increased LPS-induced leukocyte adhesion in cerebral (1247.0±125.4 cells) and mesenteric (10.2±1.0 cells) vessels of WT mice, which was reversed by oestrogen replacement to 378.9±71.4 and 6.6±2.4 cells, respectively (P<0.05 in both cases). However, in the brains (but not mesenteries) of OVX AnxA1-null female mice, oestrogen replacement potentiated both LPS-induced leukocyte adhesion (to 2047.0±378.8 cells, compared with 994.7±109.4 cells in the non-oestrogen group; P<0.05) and plasma TNF-α levels (205.00±53.11 pg/ml vs. below detectable levels in non-oestrogen group; P<0.05).

56P
Oral Communications

Glucocorticoid-induced changes in liver metabolism during mouse pregnancy


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Glucocorticoids (GCs) are used in pregnant women world-wide for treating disorders, such as asthma. This type of treatment is known to affect fetal metabolic development, but little is known about its metabolic effects on the mother. This study examined the effects of exposure to natural and synthetic GCs on hepatic insulin signalling and glycogen concentrations in pregnant mice.

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Pregnant mice were divided into 3 groups: untreated controls (CT) and 2 GC-treated groups given either corticosterone (Cort; 76 μg/g/day) or dexamethasone (Dex; 200 ng/g/day) in their drinking water for 5 days prior to tissue collection on either day 16 (D16) or 19 (D19) of pregnancy. After protein extraction and standardization, the abundance of insulin receptor (IR), insulin-like-growth factor 1 receptor (IGF-1R), total Akt (Akt), phosphorylation of Akt at Ser473 (pAkt), glycogen synthase kinase alpha (GSK-3α) and beta (GSK-3β) subunits and phosphorylation of the GSK-3 subunits (pGSK-3α, pGSK-3β) were measured by Western Blotting. Hepatic glycogen content was measured enzymatically. Data were analysed by two-way ANOVA, with age and treatment as factors.

There were no differences in protein expression of IR, IGF-1R, GSK-α or GSK-β with age or treatment. Akt abundance was significantly lower at D19 than at D16 in controls and was significantly lower in Dex than CT dams at both ages (Table 1). pAkt expression did not change with age. In Cort and Dex dams, pAkt expression was significantly higher than CTs at D16 but not D19 (Table 1). PGSK-3α and PGSK-3β did not differ with age in CT or Cort dams (Table 1). At D16 the expression of pGSK-3α and β was significantly higher in Cort and Dex than CT dams, an effect that persisted to D19 in Cort dams only (Table 1). Hepatic glycogen content was unaffected by treatment at D16 but was lower in both Cort and Dex groups than CTs at D19. It decreased with age in Dex dams only (Table 1). These data show that GC treatment alters insulin signalling and decreases glycogen storage in the liver of pregnant mice during late gestation. This has implications for maternal metabolism and the supply of nutrients to the fetus, which may consequencs long after cessation of treatment.

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C100

Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnant mice

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In early pregnancy prolonged stress exposure is associated with suppressed progesterone secretion and consequent disturbance of the pregnancy protective cytokine balance, threatening pregnancy maintenance (1). Circulating prolactin is increased and facilitates progesterone secretion, mediates implantation and is an immune regulator. We hypothesised that stress effects in early pregnant mice would be different from in virgins and would decrease prolactin secretion due to increased tuberoinfundibular dopamine (TIDA) neurone activity (dopamine inhibits prolactin). In addition, as progesterone/prolactin suppress interleukin (IL6) we expected that stress would elevate IL6 release due to depletion of these hormones. To test the effect of stress on the prolactin system, virgin and day (d)5.5 pregnant c57Bl6 mice (~20g, n=6-8) were administered lipopolysaccharide (LPS; I.P. 12.5μg) or vehicle and killed 90min later. Plasma was assayed for hormones/IL6 by RIA or ELISA and TIDA activation was analysed by double immunocytochemistry (ICC) for tyrosine hydroxylase (TH, enzyme in dopamine synthesis)/Fos expression. LPS decreased plasma progesterone and prolactin (2 way ANOVA; p<0.001 interaction) and increased IL6 (p<0.01 interaction) and TIDA activation (p<0.05 interaction) in pregnant vs. virgin mice. We then tested whether the different responses were due to altered prolactin negative feedback or to stress-induced proinflammatory cytokines. To test if pregnancy or stress alters prolactin negative feedback to TIDA neurones, d5.5 pregnant mice (n=7; previously given LPS or vehicle) were injected with ovine prolactin (1 μg/g body weight; I.P.). Brains were processed by double ICC for pSTAT5 (mediates prolactin signal transduction in TIDA neurones) and TH. Stress alone did not alter pSTAT5/TH labelling, however, after LPS prolactin treatment increased pSTAT5 (p<0.001 interaction), suggesting that LPS increases TIDA responsiveness to prolactin. This may mean that LPS increases prolactin feedback in early pregnancy. To test the effect of cytokines on the prolactin system, d5.5 mice (n=5-7) were treated with IL6 (I.P. 200ng), TNF α (I.P. 3μg) or vehicle. TNFα decreased prolactin (1 way ANOVA; p<0.001) and progesterone secretion (p<0.01) vs. vehicle, but IL6 had no effect. However, both cytokines induced TIDA neurone activation (p<0.01). As only TNFα inhibited prolactin secretion this suggests additional TNFα action in the pituitary. In conclusion, stress-induced suppression of prolactin during early pregnancy is associated with increased activation and signalling in TIDA neurones. The
increased responsiveness of TIDA neurones in pregnancy may be due to prolactin or other cytokines (e.g. IL6/TNFα).


Funded by a Society for Endocrinology Small Project Grant (UK). VJP was funded on a BBSRC Integrative Physiology Studentship.

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C102

Acetylsalicylic acid impairs early renal development in cultured embryonic metanephros through a COX-independent mechanism

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The maternal ingestion of Non-steroidal Anti-Inflammatory drugs (NSAIDs) during pregnancy has increased over the past few decades and has been widely associated with renal failure and abnormal renal development in the offspring at birth. NSAIDs inhibit the COX enzymes, which catalyse the formation of prostaglandins from arachidonic acid, hence implicating a role for prostaglandins in renal development. This study aimed to evaluate the effects of the non-selective NSAID acetylsalicylic acid (ASA), also known as Aspirin, on ex vivo embryonic kidney growth and development, and to establish whether ASA treated metanephroi can be rescued by exogenous prostaglandin E2 (PGE2). Mice, at 8 weeks of age or older, were timed mated and culled at day 12 of gestation. Embryos were quickly removed and decapitated. Whole metanephroi (n=8-12) were microdissected from the embryos and cultured for 7 days in DMEM: F-12 serum free medium supplemented with insulin, transferrin and selenium. Contra-laterals were cultured in ASA concentrations ranging from 0.04mg/ml - 0.4mg/ml. Images were taken at 5x magnification on the day of dissection (day 0) and every 24hrs thereafter. Metanephroi cross-sectional areas were measured using Image Pro Plus v5.1. Analysis through paired t-test, showed that ASA concentrations as low as 0.1mg/ml produced significantly smaller metanephroi by day 4 of culture compared to controls (P<0.025), while the "physiological" dose of 0.2mg/ml produced smaller metanephroi by day 3 of culture (P<0.05). An ASA concentration of 0.4mg/ml had a detrimental effect on size as well as structure from day 1 of culture (P<0.005). In addition, 0.2 mg/ml ASA significantly reduced the size of the zone of nephrogenesis (P<0.005), and 0.2 mg/ml - 0.4mg/ml ASA halved the number of ureteric bud branches after 48-hours of culture (P<0.005). These results suggest that ASA has dose dependent adverse effects on early stage ex vivo renal growth and development. Prostaglandin rescue experiments using PGE2 were then performed to determine whether the phenotype observed was as a result of COX inhibition and the absence of prostaglandin synthesis. Metanephroi were cultured with either 0.2mg/ml or 0.4mg/ml of ASA for the first 2 days of culture, and then media replaced for the remainder of the experiment with either 10μM PGE2 alone, or in combination with each ASA dose. PGE2 alone rescued metanephros back to control size by 7 days of culture when treated for 2 days with either 0.2mg/ml or 0.4mg/ml ASA. However, metanephroi cultured with PGE2, in combination with either ASA dose for the full 7 days in culture did not rescue metanephros size. In conclusion the adverse affects of ASA on early renal development may be occurring through a COX-independent pathway, and future work will focus on elucidating the possible mechanisms.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C103

Skeletal muscle deoxygenation during respiratory fatigue in heart failure

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Respiratory muscle fatigue is a key factor in the diminished exercise capacity of patients with heart failure (HF) (1,2). In addition to the ventilatory limitation in promoting hematosis, respiratory fatigue activates the respiratory muscle metaboreflex increasing reflex sympathetic outflow, which in turn limits the increase in perfusion to working muscles (2). The metaboreflex is triggered by the accumulation of metabolites within muscle caused by oxygen demand-delivery mismatch, but the effect of respiratory fatigue on respiratory muscle oxygenation in HF has not investigated. We hypothesized that respiratory muscle fatigue in HF occurs with deoxygenation of intercostal muscle leading to reflex peripheral vasoconstriction and tissue deoxygenation. Five subjects with HF and respiratory weakness (age: 66±12 yrs, left ventricle ejection fraction 34±3%; maximal inspiratory pressure <70% predicted) and nine paired healthy controls (age: 65±13 yrs) underwent respiratory fatigue induced by 3 min ventilation against fixed resistance at 60% maximal inspiratory pressure (Threshold Inspiratory Muscle Training, Heathscan Products Inc., Nj, USA) followed by respiratory unloading (3 min with non-invasive ventilation support in continuous positive airway pressure mode CPAP at 20 cmH2O) (3), while muscle oxygenation were monitored by near-infrared spectroscopy (NIRS; Oxiplex TS, ISS, IL, USA) with transducers placed on the seventh left intercostal space and on a resting peripheral muscle group (forearm). The difference in the tissue absorbancy between the 850 nm (oxy-hemoglobin) and 760 nm (desoxy-hemoglobin) wavelengths indicates tissue oxygenation. The responses to respiratory fatigue in controls were discounted from those in HF patients. Values are means±SEM, compared by two-way ANOVA-Bonferroni. Tissue oxygenation in intercostals muscle decreased during fatigue in HF relative to controls (1 min: -1.35±1.6 a.u.; 2 min: -2.23±1.9 a.u.; fatigue: -4.92±2.6 a.u., p<0.05). Forearm tissue oxygenation did not change in controls (p>0.05), but decreased in HF (1 min: -0.61±1.6 a.u.; 2 min: -1.52±1.5 a.u.; fatigue: -4.54±1.5 a.u., p<0.05). All these changes in tissue oxygenation were reversed by CPAP applied on the recovery period. Since the difference between tissue oxy-hemoglobin and deoxy-hemoglobin is considered a proxy of tissue oxygenation, reflecting the balance between oxygen delivery and utilization (4,5), the results suggest that respiratory fatigue in HF causes relative ischemia in intercostal and peripheral muscles. Therefore, these results provide original evidence that respiratory muscle fatigue provokes a local oxygen demand/delivery mismatch and tissue deoxygenation in HF, which is associated to reduction in tissue oxygenation also in peripheral muscles at resting conditions, suggesting the operation of respiratory muscle metaboreflex.
Dietary nitrate supplementation reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia

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For the same metabolic rate, there is a greater muscle metabolic perturbation (e.g., greater fall in [PCr]) during exercise performed in hypoxia compared to normoxia. It has been proposed that plasma nitrite, which can be markedly elevated by dietary nitrate consumption, may be reduced to nitric oxide under hypoxic conditions, thereby enabling a more precise local matching of blood flow to metabolic rate (1). We therefore hypothesised that the reduction in exercise tolerance that is typically observed in hypoxia compared to normoxia would be attenuated when hypoxic exercise is preceded by dietary nitrate intake. Following ethical approval, 9 subjects (2 female; mean±SD age 28±7 years) were studied on three occasions in a double-blind, randomised crossover design. The experimental protocol consisted of a 24 s bout of high-intensity knee-extension exercise for the assessment of [PCr] recovery kinetics (an index of mitochondrial function) and, after 6 min of rest, a bout of continuous severe-intensity exercise performed to the limit of tolerance (Tlim) inside the bore of a 1.5 T superconducting magnet. Subjects completed one trial in normoxia (21% O₂; CON) and two trials in hypoxia (15% O₂). During 24 h prior to the hypoxic trials, subjects consumed 0.75 L of nitrate-rich beetroot juice (9.3 mmol nitrate; H-BR) or 0.75 L of nitrate-depleted beetroot juice (0.006 mmol nitrate; H-PL). Muscle metabolism was assessed during and after exercise using calibrated ³¹P-magnetic resonance spectroscopy, and pre-exercise plasma [nitrite] was measured using a modified chemiluminescence technique. Data were analyzed using one-way repeated measures ANOVA with significance accepted at P<0.05. Plasma [nitrite] was elevated (P<0.01) following BR (194±51 nm) compared to PL (129±23 nm) and CON (142±37 nm). Tlim was reduced in H-PL compared to CON (393±51 nM) compared to PL (129±471 nM; P<0.05) but was not different between CON and H-BR (477±200 s). The end-exercise [PCr] was not different between conditions, but the overall rate of decline in [PCr] was greater (P<0.01) in H-PL (63±28 [μmol.L]⁻¹) than in CON (48±24 [μmol.L]⁻¹) and H-BR (48±21 [μmol.L]⁻¹). The PCr recovery rate constant was reduced (P<0.01) in H-PL (2.2±0.4 min⁻¹) compared to CON (2.7±0.6 min⁻¹) and H-BR (2.6±0.5 min⁻¹). Dietary nitrate supplementation reduced muscle metabolic perturbation during severe-intensity exercise in hypoxia and restored exercise tolerance to that observed in normoxia. Nitrate supplementation also abolished the reduction in the rate of PCr recovery which was observed in hypoxia, indicating enhanced muscle oxygenation and a restoration of mitochondrial function. These results suggest that dietary nitrate supplementation may have important therapeutic applications for improving skeletal muscle energetics and functional capacity in conditions where muscle O₂ delivery is compromised.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C104

The effects of prolonged hypobaric hypoxia on voluntary control of muscle in humans

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Single limb exercise in normoxia is predominantly limited by peripheral fatigue, whereas in acute severe hypoxia a supraspinal impairment of motor drive plays a greater role in exercise tolerance (1). However, the effects of prolonged exposure to severe hypoxia on supraspinal contributions to fatigue have not been investigated. Twelve healthy volunteers (male = 7) performed a 2-min maximal voluntary contraction (MVC) of the knee extensors at sea-level (SL) and following 2, 8 and 15 days living at 5050m (high altitude, HA). During the 2-min MVC electrical stimulation of the femoral nerve and transcranial magnetic stimulation (TMS) of the motor cortex were delivered intermittently. Immediately before and after the 2-min MVC, femoral nerve stimulation and TMS were delivered at rest and during submaximal and maximal voluntary contractions. Repeated measures ANOVA and Post hoc t-tests were used to compare changes between SL and HA. Partial pressure of arterial O₂ and arterial O₂ saturation were significantly decreased from SL on arrival at HA (to 44±2 mmHg and 79±3%, respectively) and remained significantly decreased at HA (P<0.01). In the non-fatigued muscle, force during brief MVCs, cortical voluntary activation (VA) and muscle contractility were unchanged during exposure to HA, but a significant decrease in M-wave amplitude (±12% ±33%; P = 0.02) and an increase in motor evoked potential amplitude (54 ±49%; P = 0.003) were observed on day 8. Following 2, 8 and 15 days at HA, force during the 2-min MVC was similar to SL values at contraction onset, but significantly lower thereafter. Fatigue index during the 2-min MVC was significantly higher in all HA trials, compared to SL (58% ± 45%, respectively; P = 0.02). The superimposed twitches evoked by TMS during the 2-min MVC were significantly larger at HA compared to SL (45 ±10 vs. 22 ±4% of pre-stimulus force, respectively; P = 0.02). The size of the twitches elicited by femoral nerve stimulation were similar between trials. Greater levels of supraspinal fatigue (measured by cortical VA) following the 2-min MVC were observed at HA compared to SL, the greatest difference being observed on day 2 at HA.
(20 ±22% decrease pre-post 2-min MVC vs 6 ±9% decrease at SL; P = 0.02). Reductions in potentiated twitch amplitude, a marker of peripheral fatigue, tended to be less following the 2-min MVC at HA (P = 0.12). These findings indicate that: 1) prolonged exposure to hypobaric hypoxia does not influence voluntary activation of muscle or force generating capabilities in resting conditions, although sarcolemmal and corticospinal excitability are affected; 2) fatigability during sustained voluntary contraction is exacerbated during prolonged exposure to hypobaric hypoxia, and 3) this additional fatigue is the result of an oxygen-sensitive source of inhibition of descending voluntary drive, possibly mediated by prolonged arterial hypoxemia.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C106

Chronic Epo deficit induces exercise performance decrease and massive muscle proteolysis in running mouse

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The protective role of Erythropoietin (Epo) on tissue is still under debate. Indeed, it was reported in brain, kidney, heart but not in skeletal muscles. Meanwhile deficit or supplementation of Epo is known to modify the exercise performance and specially aerobic metabolism. Therefore, the purpose of this study was to investigate the protective effect of Epo in muscle and to examine the relationship between this potential protective effect and aerobic metabolism improvement.

The performance of 17 mice (9 Epo-deficient (Epo-d) and 8 inbred control) was determined by running on a treadmill with oxygen uptake ( ) measurement. Mice were sacrificed 24h after a last exhaustive treadmill exercise at the critical speed (CS). The Tibialis anterior and soleus muscles were removed. Total RNA was extracted from the muscles for gene expression analysis by microarray and quantitative real time PCR validation (RT-qPCR). The hematocrit (Htc) of the Epo-d mice was reduced by 50% compared with the control group (p<0.05). Also their performance was reduced by 25 to 30 % depending on the variables: vPeak, (p < 0.01), CS (p < 0.01), and max (p < 0.001). Moreover, vPeak and max of all mice and within each group were correlated with the Htc. A total of 1583 genes exhibited significant changes of expression levels (p < 0.05) between the Epo-d and the control mice. This set contained 68 up-regulated genes (normalized ratio > 1.4), 115 downregulated genes (normalized ratio < 0.80). Most of the significant genes were moderately modulated genes (0.80 < normalized ratio < 1.4).

According to data mining analysis, the exercise in Epo-d mice induced muscular proteolysis, hypoxia and oxidative stress which may be explained by glycolysis and mitochondrial phospho-oxidation inhibition, lowered cytoskeleton components and defective intra cellular transport. These results suggest that Epo have a protective role on skeletal muscles.

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Human in vivo tendon adaptations to resistance training & detraining at different muscle lengths

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The material properties of the muscle-tendon complex (MTC) alter in relation to mechanical stress (e.g. resistance training, RT (1, 2)). However, performing exercise at different muscle lengths will alter the mechanical stress delivered to the MTC via changes to the moment arm of the in-series elastic component (3). The in vivo response of the MTC to dynamic resistance training and detraining at two different muscle lengths are yet unknown. Two training groups - LL (n=8) and SL (n=8) undertook 8 weeks of dynamic RT and 4 weeks detraining at either a short muscle length (SL) or longer muscle length (LL). Tendon dimensions (CSA and length), stiffness (K), Young's Modulus (E), antagonist co-contraction (TAnt) and maximal voluntary (isometric) muscle torque (MVC) over a range of angles (30-90o knee flexion) were measured at weeks 0, 8, 10 and 12 using ultrasonography, EMG and dynamometry. A control group (n=10) was also monitored during this period. The local Ethics committee approved this study and participants gave written informed consent. Results are mean ± S.E.M., and compared via ANOVA (alpha ≤ 0.05). There was a significant effect of training (P<0.01) in SL and LL groups in MVC, K (figure 1) and E compared to baseline and control data. There was no training effect (P>0.05) on patellar tendon dimensions or TAnt in training groups. SL increased MVCs at knee joint angles between 50-75o, whereas LL increased MVCs over a wider range (30-90o). There was a main group effect (P<0.05) in K (45±6% vs. 32±2%) and E (42±5% vs. 30±2%) at week 8, with LL exhibiting greater adaptations than SL (P<0.05). The group effect in K and E remained at week 10. Detraining resulted in significant (P<0.05) deteriorations in tendon mechanical properties measured in both groups by week 12 compared to week 8, although they remained significantly elevated relative (P<0.01) to week 0. Our results show that adaptations to the MTC are superior immediately following a period of chronic resistance training at a longer muscle length in comparison to a shorter muscle length. The relative workloads between groups were similar, with the differential mechanical stress to the unit (3) likely to form the basis for the differential magnitude of responses between LL and SL, as observed during isometric training (4). In order to determine the underlying mechanisms of these specific adaptations, an investigation into endocrine signalling is warranted, as it has been suggested that IGF-I and TGF-β1 (i.e. markers also of the potential hypertrophic response to muscle-loading induced stress) may be mediators of collagen expression in response to mechanical loading of tendon (5).


The authors are ever indebted to the study populations without whom none of this work would have been possible. The authors are also grateful for the continued support from their research institute

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Assessing students’ perception of feedback

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The national student survey (NSS) has highlighted assessment and feedback as a key area for student concern. In the 2009 NSS at the University of Birmingham, 86% of students were satisfied overall with the BMedSc programme. However, 46% of students felt feedback did not help clarify things they did not understand. A preliminary study was conducted to test the students’ perception and opportunities for feedback within a first year physiology module of the BMedSc programme in 2009-2010. Fifty four students were present during the scheduled session and completed the questionnaire.

From the data collected students generally think they do not receive feedback (65% of cohort) on their learning and understanding. However, 87% of the students considered small group teaching (SGT) sessions as an opportunity to test their understanding with 30% of the students taking questions into the SGT sessions, where all students attended at least one SGT. Nearly all students (96%) agreed the laboratory practical sessions tested their understanding with 91% of the students discussing the practical with the lab staff during the session. Questions were provided within the practical schedule to test the students understanding; 78% of the students attempted the questions but only 31% sought feedback on the questions answered. Of the 54 students, 69% agreed the specific module discussion board available via the webCT VLE tested their understanding. However, less than half of the students accessed the generic feedback posted online relating to their course assessment; 79% of those that did correlated the feedback given to their own performance.

These preliminary results imply there is a discrepancy between the students’ perception of receiving feedback and their known opportunities to attend, engage and ask questions where their understanding can be tested. Rather than provide more opportunities for feedback (which has implications for the workload of the academic and student timetables), it is important to ensure that students understand what feedback is, what is being currently provided and how they can use it effectively.

National Student Survey 2009. URL: http://www.hefce.ac.uk/learning/nss/

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OeRBITAL: Open educational resources for Bioscientists Involved in teaching and learning

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Open educational resources are digitised resources which have been made freely available by their creators, through Creative Commons licensing, for use by both students and academic staff in their research and teaching. Open educational resources can include entire courses, course materials, videos, problem solving exercise and many other types of resources. The use of open educational resources is increasing in popularity amongst Higher Education Institutions. Many Learned Societies and Professional Bodies are also promoting their use and dissemination within their disciplines. Indeed, the Physiological Society has its own repository of teaching materials, Philter1 (Physiologists’ Image Library & Teaching Resource) which is available to its members.

OeRBITAL2 (Open educational resources for Bioscientists Involved in teaching and learning) is a Joint Information Systems Committee (JISC) and Higher Education Academy-funded UK Centre for Bioscience project, the aims of which are to discover and promote the use of open educational resources in the Biosciences. Ten Discipline Consultants, who collectively have expertise from across the biosciences, have been appointed. These Discipline Consultants are searching through existing repositories, evaluating individual resources, and disseminating information on the best of these within their discipline via the project wiki and through links with Learned Societies. Here, we present examples of resources and repositories for Physiology linked material including Topics in Physiology3, and the Life Sciences Search Engine, Vadlo4.

The benefits of sharing teaching resources with colleagues outside of your own Institution include the promotion of your own teaching or institution and enhancing your students learning experience by giving them access to materials or resources that you are unable to provide, particularly if these utilise the knowledge or expertise of colleagues which is not available within your own Institution. There is also the potential for enormous savings in cost and time; providing excellent resources for your students without reinventing the wheel. Furthermore, through the use of Creative Commons or other licensing, users can modify and potentially improve or enhance your resource for the community whilst retaining acknowledgement of your original contribution.

1Physiologists’ Image Library & Teaching Resource http://www.physoc.org/site/cms/contentviewarticle.asp?article=904
2Open educational resources for Bioscientists Involved in teaching and learning (http://heabiowiki.leeds.ac.uk/oerbital/)
3Topics in Physiology http://virtuallabs.stanford.edu/demo/
4Vadlo http://Vadlo.com

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Young people’s perceptions of the use of animals in scientific and medical research in the UK

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Surveys of the UK adult population have shown that 70% agree with the use of animals in medical research (MORI, 2010). However, there are no comparable studies of young people. Prior to 2006, discussion of the use of animals in research and the ethical issues arising from such use was included in the Religious Studies and Philosophy GCSE specifications. Post 2006, with the increased emphasis in science GCSEs on how science works, ethics, including the ethics of animal experimentation, became part of all science and biology GCSE curricula. Subsequent revisions for 2011 onwards have lead to a reduced

PC1

PC2

PC3
emphasize or exclusion of this important topic. Given that current teenagers will become future voters and therefore be able to influence any changes in animal welfare legislation, the aim of this study was to determine young people’s views on animal experimentation.

A short 20 minute presentation followed by the opportunity for students to ask questions on the use of animals in research was delivered in secondary schools and colleges within West Yorkshire. Electronic voting handsets were utilised to gather student opinions anonymously before, during and after the session.

The seminar was delivered to 466 Year 8 to Year 13 science students from 11 schools. These schools included select grammar, comprehensives, those in economically deprived areas, faith and independent schools. The majority of students (78%) had never or only occasionally thought about the use of animals in research before the seminar, with only 37% either agreeing or strongly agreeing with their use. After the session, the level of acceptance had increased to 66% (p < 0.01, student t-test). When asked how new medicines should be developed and tested, 31% thought that non-animal (alternative) experimental preparations should be used, 24% would utilise animals whilst 22% would use prisoners in the first instance. Students also had serious misconceptions about practices in research laboratories, for example, 53% thought that research animals were kept in small confined cages. 26% of students also thought it was acceptable for those opposed to animal experimentation to use any means to prevent such research.

This study demonstrates the need for scientists, particularly those who use animals or animal tissues in their research, to engage in outreach activities in schools in order to provide young people with unbiased information on the use of animals in scientific and medical research. These outreach activities would enable young people to make an informed decision for themselves as to whether this use of animals can be justified.


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**PC4**

**Evaluating student perceptions of a module: a modified nominal group technique**

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The modular structure of the MBChB course can lead to compartmentalization of learning. The ability to understand, interpret and integrate information is essential for medical students while they are studying and in their future careers as doctors. In Birmingham, the pre-clinical Integrated Problems (IP) module was developed with the aim of integrating learning across the curriculum. The module uses medical case studies for the students to learn in an enquiry-based setting in order to develop these important skills as well as to develop their presentation, group learning and reflective skills.

At present the main form of feedback from students comes from a standard College of Medical & Dental Sciences module feedback questionnaire consisting of general questions scored on a Likert scale and free text comments. One of the comments asks the students to list the three best things about the module, however with a cohort of ~400 students this information is difficult to collate and interpret. The aim of this study was to design an activity to collect representative data from the students in a form that was easier to analyse.

A modified nominal group technique activity was developed to collect this data. In tutorial groups (n=22), students (16-18 per group) were asked to take 5-10 mins to discuss, list and rank what they perceived to be the three best things about the IP module. Students were told they must reach a consensus as a group. The activity was performed at the beginning of semester 1, year 2 when students were in new tutorial groups with new facilitators; students were asked to reflect on their experiences in year 1. Ranked lists were returned for analysis. Each of the three best things were assigned to ten representative categories and given points depending on their rank (1st=3, 2nd=2, 3rd=1). An Importance Rating (IR) was calculated by multiplying the number of mentions by the total score in each category.

21 out of the 22 (95%) tutorial groups provided a response to the activity. Using the IR the best thing about the module was the opportunity for team-working and getting to know their tutorial group (IR 351;13 mentions, 27 points), followed by developing confidence (IR 253; 11 mentions, 23 points) and choice of what to study (IR 209; 11 mentions, 19 points). There was a clear gap between the cohort’s three best things and the fourth ranked category (developing presentation skills, IR 112).

This study introduces an activity for collecting feedback on a specific component of a module and a method for analysing and ranking the feedback. Not only is this technique aligned with the learning outcomes of the IP module but also allows representative data to be easily collected from a cohort of ~400 students. This technique could be easily adapted to collect feedback on a huge variety of teaching and learning activities.


Prem Kumar & Jane Norton.

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**PC5**

**Developing skills for the future: An alternative team-based final year biosciences enterprise project**

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In response to increasing pressure on traditional ‘wet’ laboratory projects from rising student numbers, we introduced the Life Sciences Enterprise Project (LSEP) in 2006 as an alternative project option. Working in teams of 6, students use research knowledge outputs from life sciences to produce a business plan for a real product or service. In addition to developing commercial awareness, these students also develop a wide range of transferrable skills to enhance employability.
In semester 1, students select a project ‘theme’, e.g. brain development; and an ‘avenue’, e.g. the role of folate in neural development. With supervisor support, each student produces a literature review focusing on the scientific content of their avenue. With a deeper understanding of their field, students develop an idea for a product or service relating to their research. Assessment of the feasibility of their idea is then presented to the year group in a poster event attended by entrepreneurial partners. Each team decides which idea has the greatest potential for successful commercialisation and will form the basis of the team business plan in the following semester. In semester 1, workshops provide an overview of the processes underpinning the entrepreneurial journey from an initial idea to a successful venture and also of the skills required for effective team working and project management. The students have access to business advisors in semester 2, but in essence the project is driven by the students in an enquiry-based learning (EBL) approach. The project culminates in our ‘Lion’s Lair’ event where students pitch their idea to a panel of business professionals.

One of our key aims was for students to develop a wide range of transferable skills to enhance employability. We used the 5-level Likert scale to assess student’s perception of the skills developed or acquired during LSEP. Values are presented as mean±S.D; n=36. Of particular note, students agreed that LSEP had helped them to develop problem-solving skills (4.3±0.7), decision-making skills (4.4±0.7), formal oral communication skills (4.7±0.5) and the confidence to use their own initiative (4.3±0.6). Teamwork is a prominent feature of this project, comprising 70% of the final mark. Students agreed that their team building skills had also improved (4.7±0.5). Student comments included: “it has allowed me to develop many existing transferable skills as well as acquiring new valuable skills”; “I was able to use LSEP as evidence at interview as these are the competencies expected by employers”.

This project approach enables our students to apply their subject-specific knowledge in an applied way to an authentic problem and develop a wide range of transferable skills in preparation for employment.

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PC6

Reversing the drain – educating the next generation of in vivo physiologists
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Practical in vivo skills are a vital resource that must be maintained to enable the UK to remain internationally competitive in the biosciences. Once the mainstay of Physiology and Pharmacology teaching, practical in vivo experimentation is now absent from most degree courses as fewer universities have the staff or resources required to teach at this level. In a drive to halt the attrition, a consensus report by stakeholders from Academia and the Pharmaceutical Industry recommended establishing as a priority “taught Masters degrees that target ‘hands-on’ training to those most likely to use the skills in future courses or careers” (1).

In response, the University of Birmingham established a new MRes (in vivo) degree in 2010, which stands alone or forms the first year of 1+3 doctoral training programmes. The current intake is funded through a BBSRC Masters Training Grant and internally funded studentships. It is also open to self-funded students. Our programme places a heavy emphasis on the practical as well as the intellectual mindset required for in vivo research, with an emphasis on active and experiential learning through the use of varied educational activities.

Training for a Home Office licence exposes students to the ethical and legal requirements of in vivo research. Thereafter, in a dedicated laboratory they develop core skills via a progression 3 month long self-directed programme of mini-projects. There are no practical work sheets. Students design their own experiments and set up equipment etc. They develop proficiency in making simultaneous recordings of a range of physiological variables, recognising artifactual data, trouble shooting, data analysis, report writing, presentation skills etc. Manual dexterity improves, the students develop a more critical sense of observation, a respect for living tissue and the concept of ‘keeping many balls in the air’. Starting with human subjects they move to ex vivo amphibian and finally mammalian preparations. Staff input is tapered as they become more proficient, but progress is monitored closely and feedback offered in weekly ‘roundup’ sessions.

Running in parallel, a theoretical In vivo Techniques module is delivered by research active staff. Generic skills are fostered by our common Graduate School Lecture series, a journal club, and experimental design/data analysis assessments. Students are encouraged to attend relevant research seminars and to interact closely with the in vivo research community. A Taster Projects module gives them direct experience of a wide range of ongoing in vivo research in mammals (15 different projects in 2010-11) before choosing a final in-depth 20 week research project, which culminates in a substantial dissertation and vivace examination.

Student feedback indicates an appreciation for the breadth of practical experience, coupled with intensive tuition in theoretical aspects.

(1) In vivo sciences in the UK: sustaining the supply of skills in the 21st century. ABPI/Biosciences Federation, 2008.

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PC7

Why is undergraduate lecture attendance so poor?
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Teaching staff often complain about poor undergraduate lecture attendance. Why do a significant number of students fail to attend lectures on a regular basis? In addition, have student attitudes towards lectures changed over recent years? In an attempt to address these questions and to try and understand undergraduate student attitudes towards lectures this study compares the findings obtained from two separate student questionnaires – one conducted in 2003, with one conducted in 2010. Although the questionnaire was made available to students studying modules in physiology, it was made clear to participants that questions were designed to determine their attitudes towards lectures in general, not just those delivered as part of each module.

A paper-based questionnaire was presented to students in 2003 studying a level 3 (3rd year of a 4 year BSc(Hons) programme)
Physiology module, ‘Human Systems Physiology’ (HSP). In 2010, the same set of questions were provided using an online questionnaire students studying HSP and to level 2 students studying the module ‘Human Form and Function’ (HFF). Responses to individual questions ranged from n=33-89 from which percentage responses were determined and ranked. When asked to select the main reason for missing lectures, ‘Lack of self discipline’ was the most popular choice for students studying HSP in 2003 (26%) and HFF in 2010 (35%). ‘Lectures are not stimulating enough’ was the first choice for HSP 2010 (36%) and second most popular choice for HFF 2010 (21%), compared to 0% response from HSP 2003 students. This tends to suggest that current students do not engage to the same extent with lecture-based delivery as their peers did in 2003.

Interestingly, ‘I have to work to earn money’ was the second most popular response to the same question for HSP 2003 (26%) but, despite the perceived increase in cost of studying at University over recent years, ranked as third-most popular reason for HSP 2010 (15%) and HFF 2010 (18%). Although all groups were in agreement that ‘PowerPoint files should be put on the module virtual learning environment before the lecture’, HSP 2003 students felt ‘Lectures should be compulsory’, whereas HSP 2010 and HFF 2010 students took the opposite view that ‘Lectures should not be compulsory’.

Overall, students surveyed did appreciate the importance of lectures as a means of obtaining curricular content and that non-attendance decreases their chances of passing exams. Despite this, there now seems to be an increased perception that lectures are not stimulating enough. As education practitioners undergraduate teaching staff have to ensure lectures are made interesting and interactive and form part of a blended-learning approach to delivery of physiology.

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PC8

Preclinical Course Preferences, Perceptions and Satisfaction

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No studies have assessed preclinical students’ preferences for their courses, namely, anatomy, physiology & medical biochemistry, their perceptions of these courses in terms of ability to understand the subjects, ability to interact with the lecturers involved in the teaching of these courses & their perceptions of the relevance of these courses to the medical curriculum. This study compared the preferences, perceptions & satisfaction of 300 level female preclinical medical & dental students of the University of Benin, Nigeria, with those of their male counterparts. The three courses assessed were anatomy, physiology & medical biochemistry. A structured questionnaire was used to elicit relevant information. Results were expressed as percentage & means ± S.D. & presented as frequency distribution tables. Student’s t-test was used to calculate the differences between the means. A P<0.05 was taken as statistically significant. Of the 310 respondents, 90 (29.0%) were females studying medicine (Fem-med); 167 (53.9%) were males studying medicine (Male-med); 14 (4.5%) were females studying dentistry (Fem-dent), while 39 (12.6%) were males studying dentistry (Male-dent). Their ages ranged from 19–37 yrs, with a mean age of 23.0 ± 0.7 yrs. Although majority of the Fem-med were most confident of passing anatomy (47.5%) and physiology (37.5%), they had very little likelihood of considering any of these preclinical courses as specialties after graduation. A larger % of males than female respondents were likely to consider specializing in any of the three courses, with anatomy (39.5%) & physiology (53.5%) being the preferred courses to consider as specialties. Dental students were more likely to consider choosing any of the three courses as specialties after graduation than medical students (P<0.05). Majority of students perceived anatomy & physiology as the most relevant to the medical curriculum, while medical biochemistry was considered to be the least relevant (P<0.05). All students perceived anatomy as being the most difficult to understand, but however claimed to have the most interaction with anatomy lecturers. Like the males, majority of the female students felt that they were most satisfied with the quality of teaching (52.6%) & of practicals (46.3%) in physiology. In conclusion, although there are gender differences in preclinical course preferences & the willingness or otherwise to specialize in them after graduation, students’ perceptions & satisfaction in the courses are generally similar.

Male - dent preference for courses, in relation to items listed

PC9

A simple method for the ongoing evaluation of standard setting for one best answer exam questions

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Introduction: Standard setting methods are now routinely used to determine passing scores in multiple choice examinations. However it is often difficult in everyday practice to know whether the standard setting process is reaching good judgements about the performance of borderline students. A new

<table>
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<th>PHYSIOLOGY</th>
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A life coaching approach that keeps students on track

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One of the essential qualities for student achievement and success is persistence, yet motivation to accomplish project and/or assessment tasks can sometimes be complex. Some student behaviours present a continual challenge for tutors who are striving to support independent learning and creative problem solving e.g. through projects and assessments (Di Carlo, 2009). Moreover, being on the receiving end of criticism, albeit well-intentioned, can leave students feeling vulnerable, defensive and even ashamed about lack of progress (Francis and Woodcock, 1996). An oft-heard response to this situation is 'I'll try' which indicates that s/he is willing to make an effort of some kind but which also denotes a degree of ambivalence or lack of commitment. A life coaching approach (Neenan and Dryden, 2002) was therefore adopted to improve motivation amongst students.

A simple proforma was developed to provide a framework that has been used during academic and personal tutorials with more than 300 students taking a range of courses in physiology and health sciences. The majority were able to identify individual challenges and unique learning outcomes through the use of structured prompt questions. This stage also helped to discuss barriers that hindered progress. Targets were set by each student who also identified a personal incentive for meeting with decision-making is proposed. The goal-setting approach appears to elicit greater levels of student enthusiasm and supports decision-making while the review process helps to ensure accountability for desired actions. The life coaching strategy therefore appears to facilitate deeper appreciation of the implications of persistence and action by encouraging individual learners to take greater responsibility for the consequences of their activity or inactivity.


The effects of closer integration of Nursing and Bioscience departments on student attitudes to a first year Biomedical Science course

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A knowledge of the Biomedical Sciences is a well-established requirement for the modern Nurse, such knowledge providing an understanding of the Scientific basis of Nursing practice (Trnobrański, 1992). The teaching of Biomedical Science, however, presents a number of challenges, particularly since Nursing Education transferred from a chiefly hospital base to a University setting (Carlowe, 2005). Chief among these challenges is relating Biomedical Science to Nursing Practice. It has been suggested that the best results are achieved when registered Nurses and Biomedical Scientists teach together in a “teaching partnership” (Larcombe and Dick, 2003). In such a partnership, Biomedical Scientists appreciate the context and relevance of concepts being discussed while Nurses benefit from the scientific rigour inculcated during this phase of teaching.

Between September 2008 and April 2010, we set up a “Teaching Partnership” (TP) arrangement between the Centre for Biomedical Science Education and the School of Nursing and Midwifery in the Queens University of Belfast. Staff members teaching Biomedical Science and Nursing Practice visited each other’s classes to ascertain how best to relate the subjects. After this, changes were made to all classes in each subject reflecting what had been learned. Student attitudes in groups attending Biomedical Science classes before, during and after the TP process were assessed by questionnaires rating the relevance of the subjects to each other and their careers. A 5 point Likert scale was used to evaluate the student response to each of the questions with 5 indicating strong agreement with a statement and 1 strong disagreement. In addition to
this, there were 3 open ended questions focussing on the relevance of the subjects to their career. Ratings are given as mean mark out of 5 ± S.E.M, n = 110, 154 and 180 for groups before, during and after TP respectively. Students responded enthusiastically in all questionnaires about both subjects with scores over 3 for all positive statements from groups assessed before, during and after implementation of TP. Scores in questions assessing the link between Nursing practice and Biomedical Science improved significantly while TP was being implemented (going from 3.7 ± 0.1 to 3.9 ± 0.1), but fell back again to 3.6 ± 0.1 after the process was complete (p < 0.05 ANOVA). Similar significant patterns were seen in questions asking about how Nursing practice aids understanding of Biomedical Science and vice-versa, with highest scores recorded while staff visited classes in the other discipline. We conclude that for TP to work, staff from the different topics must be visible in the classroom. Changes made in subject material taught are not sufficient.


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PC12

The effect of vitamin B12 status and supplementation on autonomic nervous activity in a healthy elderly population

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Vitamin B12 deficiency is a worldwide problem. There is no population data on vitamin B12 status in elderly Indians but the extent of the problem is likely to be at least as high, if not higher, than the prevalence reported in western countries for a variety of reasons. Present study objective was to assess cardiac sympathetic and parasympathetic activity using heart rate variability (HRV) in elderly individuals of low and high vitamin B12 status and to study the effect of vitamin B12 supplementation in those with low vitamin B12 status.

140 healthy elderly subjects aged >60 years were screened and 48 subjects fitted the criteria. They underwent blood sampling, HRV, nerve conduction and cognition assessment (Montreal Cognitive scoring). Nerve conduction assessment was performed on the median nerve and included both motor and sensory assessment (RMS EMG EP machine, Recorders and Medicare systems, Chandigarh, India). For motor nerve conduction studies, surface electrodes were used. For orthodromic sensory nerve conduction studies, surface ring electrodes were used. Evaluated variables included the latencies, conduction velocities and amplitude. Subjects were classified based on their age (<65 years and ≥65 years) and based on median plasma vitamin B12 level (118 pmol/l) into lower vitamin B12 and higher vitamin B12 groups.

Low frequency (LF) HRV presented in absolute units was significantly higher in the high vitamin B12 group when represented as a complete group. When stratified by age, among <65 years age group, high vitamin B12 group demonstrated a significantly greater response. About 9% of the variability in LF was explained by vitamin B12 status. Post supplementation LF and HF (high frequency) HRV in absolute units and total power were significantly raised as compared to pre-supplementation values when analyzed as a complete group and in < 65 years age group. In conclusion evaluation of vitamin B12 deficiency and its supplementation is best done in “younger” elderly individuals in order to achieve enhanced effects on the autonomic nervous system.


St John’s Research Society, Bangalore.

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PC13

Neurons from the cardiovascular region of the nucleus tractus solitarius target GABAergic and presynaptic neurons of the paraventricular nucleus of the hypothalamus

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The paraventricular nucleus of the hypothalamus (PVN) and nucleus tractus solitarius (NTS) are critical components of the neural circuit that controls the cardiovascular system1. Presynaptic neurons of the PVN regulate sympathetic activity, thus contributing to cardiovascular control. These neurons receive information about cardiovascular status from neurons in the NTS2. Furthermore, transynaptic labelling has shown the presence of GABAergic interneurons that modulate presynaptic PVN neuronal activity3. The question therefore is; do the GABAergic interneurons also receive an NTS input providing a further pathway to control sympathetic activity? This is important because elevated sympathetic nerve activity, strongly associated with cardiovascular disease, is partly generated from the presynaptic PVN neurons1. This study used anterograde (Biotin dextran amine-BDA) and retrograde (Fluoro gold-FG or cholera toxin subunit B-CTB) tracing with immunohistochemistry for GABAergic neurons to identify the terminal neuronal targets of the ascending projection from the NTS.

Experiments were performed on male Wistar rats (n= 6) in accordance with the Animals (Scientific Procedures) Act, 1986. For labelling of NTS projecting axons, animals were anaesthetised i.p. with medetomidine (0.25 ml/100g) and ketamine (0.06 ml/100g). Animals received an injection of 10% BDA into the NTS (R/C 13.68 mm, L 0.5 mm, D/V 8.00 mm)4. After a 14 day recovery period, animals were re-anaesthetised (as previously described) to inject 2 % FG or 0.5 % CTB into the spinal cord2. Post-operatively, the animals were administered buprenorphine (0.1 ml/kg). Following a further recovery period (7-14 days) animals were humanely killed (pentobarbital 60 mg/kg i.p.) perfused fixed (4 % paraformaldehyde-PFA or 4 %
PFA and 0.5% glutaraldehyde) with removal of brain and spinal cord. Frozen sections were processed to reveal BDA, CTB and GABAergic interneurons in the PVN. Tissues were examined under bright field or epifluorescence for the existence of putative connections between the NTS and GABAergic interneurons and presympathetic neurons of the PVN.

Ascending axons from the NTS coursed through and around the PVN nucleus. The NTS terminal axons showed numerous varicosities, some of which appeared to closely appose the somata and dendrites of presympathetic neurons and the GABAergic neurons adjacent to the PVN.

We have confirmed and extended our earlier finding such that axonal fibres from the NTS target presympathetic neurons of the PVN and GABAergic interneurons surrounding the PVN. Functionally, this neural circuit underpins the regulation of sympathetic outflow and therefore has the potential to contribute to the generation of abnormal sympathetic activity by the PVN.


We acknowledge the British Heart Foundation for funding.
Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC14
Effects of respiratory loading on global and stimulus evoked cerebral blood flow
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To use functional magnetic resonance imaging (FMRI) to understand neuronal processing of respiratory disorders it is vital to understand the effects of alterations in intrathoracic pressure on cerebral blood flow (CBF). We investigated how resistive inspiratory and expiratory loading affect CBF and the stimulus-evoked CBF response. We studied 13 healthy volunteers (11 male; age 28+/−7) twice, first in a physiology laboratory, then in a 3 Tesla MRI scanner. Resistive loads were applied via a custom designed breathing system. Breathing room air, subjects were trained to maintain their end-tidal carbon dioxide (PETCO2) constant via visual feedback. 270-second blocks of inspiratory loading (+10cmH20), expiratory loading (+10cmH20) and no loading were alternated and a 2Hz flashing checkerboard was shown for 120 seconds every 240 seconds. Session 1: middle cerebral artery velocity (MCAV) and blood pressure were continuously recorded. Session 2: CBF perfusion was acquired with a whole-brain pseudo continuous arterial spin labeling (ASL) sequence. Data was analysed in FSL and Matlab. Session 1: We observed a transient CBF increase (first 30 seconds) after application of inspiratory load (Figure 1) and a simultaneous increase in PETCO2, but no change in mean arterial pressure (MAP) or heart rate (HR, Table 1). Removal of inspiratory load and application of expiratory load caused no changes in CBF, MAP and HR. Session 2: Voxel wise analysis shows significant bilateral CBF increases in the visual cortex to visual stimulation (25.2%+/−37.1) and no interaction of visual activity with inspiratory or expiratory load. Inspiratory and expiratory loading caused no global changes in CBF. After a transient increase PETCO2 was maintained at 5.5% (+/−0.8). We show transient effects of respiratory loading on CBF in the first 30 seconds of application of inspiratory resistance. No significant global effects of respiratory load on CBF or on the stimulus evoked CBF response occurred over 270 seconds. Hence, longer applications of respiratory load can overcome potential confounds of transient CBF changes. FMRI is hence a useful tool for looking at brain mechanisms of respiratory conditions, either during external resistive loading or in disease conditions associated with altered airway dynamics.

Table 1 Physiological data after application of inspiratory loading

<table>
<thead>
<tr>
<th>Time (seconds)</th>
<th>Baseline</th>
<th>0-5</th>
<th>5-10</th>
<th>10-15</th>
<th>15-20</th>
<th>20-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAV [cm/sec]</td>
<td>86.84</td>
<td>96.84</td>
<td>96.84</td>
<td>96.84</td>
<td>96.84</td>
<td>96.84</td>
</tr>
<tr>
<td>MAP [mmHg]</td>
<td>71 (5)</td>
<td>71 (5)</td>
<td>71 (5)</td>
<td>71 (5)</td>
<td>71 (5)</td>
<td>71 (5)</td>
</tr>
<tr>
<td>(CO2) [%]</td>
<td>5.54 (5)</td>
<td>5.54 (5)</td>
<td>5.54 (5)</td>
<td>5.54 (5)</td>
<td>5.54 (5)</td>
<td>5.54 (5)</td>
</tr>
</tbody>
</table>

Mean(SD), significance: * p<0.05, ANOVA

This study is supported by the Medical Research Council, UK. AH is supported by the DAAD, Germany.
Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC15
Sympathoexcitation induced by optogenetic stimulation of astrocytic networks within the brainstem is partially mediated by glutamatergic mechanisms
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Astrocytes control complex central nervous functions by regulating synaptic strength and information processing (Halassa et al., 2007). Optogenetic activation of astrocytes located in
the rostral ventrolateral medulla (RVLM) produces significant elevations in sympathetic nerve activity and blood pressure (Marina et al., 2010). Here, we sought to identify the putative gliotransmitter involved in glial control of central sympathetic tone. Experiments were conducted under the UK Animals (Scientific Procedures) Act 1986. Adult male Sprague Dawley rats were anaesthetised with a mixture of ketamine (60 mg kg\(^{-1}\); i.m.) and medetomidine (250 μg kg\(^{-1}\); i.m.) and were stereotaxically targeted with an adenosinergic vector to express mutated channelrhodopsin2 -ChR2(H134R) - in the RVLM under transcranial control of the enhanced astrocytic-specific GFAP promoter (Liu et al., 2008). Experiments were carried out 10-12 days after viral transduction under α-chloralose anaesthesia (100 mg kg\(^{-1}\) iv, supplemented with 20 mg kg\(^{-1}\) iv as required). Animals were ventilated artificially with O₂-enriched air, the ventral surface of the brainstem was exposed and renal sympathetic nerve activity (RSNA) and mean arterial blood pressure (MABP) were recorded. The ventral medullary surface was stimulated unilaterally with pulses of blue light (20ms duty cycle) after application of artificial cerebrospinal fluid (aCSF, i.e., control), PPADS (100 μM), MRS2179 (200 μM) or AP-5 (500 μM) onto the ventral medullary surface. RESULTS: Sympathoexcitatory responses to photostimulation of ChR2-expressing RVLM astrocytes were unaffected by application of purinergic receptor antagonists: RSNA increased by 19.4 ± 1.5 % (aCSF, n=7) vs 19.7 ± 4.8 % (PPADS, n=3) and 24.6 ± 5.6 % (MRS2179, n=4); MABP increased by 13.8 ± 3.1 mmHg (aCSF, n=7) vs 18.5 ± 7.5 mm Hg (PPADS, Ttest=0.4; n=3) and 19.4% ± 8.7 mmHg (MRS2179, Ttest=0.2; n=4). However, sympathoexcitatory responses to optogenetic stimulation of RVLM astrocytes were substantially decreased in the presence of NMDA receptor antagonist: RSNA increased by 48.2 ± 17.4 % (aCSF, n=4) vs 21.8 ± 14.8 % (AP-5, n=4). However, pressor responses were not affected by AP-5: MABP increased by 12.2 ± 3 mmHg (aCSF) vs 7.6 ± 0.5 (AP-5, Ttest=0.1, n=4). Immuno-histochemical analysis revealed abundant ChR2-expressing astrocytes located in close proximity to tyrosine hydroxylase-positive neurons within the RVLM. CONCLUSIONS: These data suggest that glutamatergic mechanisms mediate, at least in part, the activation of local pre-sympathetic neuronal networks of the RVLM in response to optogenetic activation of local astrocytic networks. The role of purinergic gliotransmission in sympathoexcitation is still under scrutiny. Marina N et al. (2010). Proc Physiol Soc. 19, C77. Halassa MM et al. (2009). Neuropharmacology, 57, 343-346. Liu B et al. (2008). BMC Biotechnol. 16:849. This work was supported by the British Heart Foundation. AV is a Wellcome Trust senior fellow.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Diurnal variation in the mechanical and neural components of the baroreflex

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Diminished baroreflex gain is a marker for cardiovascular risk (La Rovere et al., 1988). The incidences of myocardial infarction, sudden cardiac death and stroke are highest between 0600 and 1200h, and yet the mechanisms behind this diurnal variation remain unclear. Our aim was to determine the contribution of the mechanical and neural components of the cardiac baroreflex to diurnal variation in blood pressure (BP) control. Given that diurnal variation has been reported in carotid arterial distensibility (Kool et al., 1991), we hypothesised that time-of-day differences in baroreflex gain are explained by variability in the mechanical component. In 7 men and 5 women (mean ±SD age 24.7 ±4.0 yrs), we measured continuous BP, R-R intervals and carotid artery diameter during intravenous bolus injections of sodium nitroprusside (SNP) followed by phenylephrine (PE) (modified Oxford method) at 0700 and 1600h. The modified Oxford technique quantifies baroreflex gain for falling BP via SNP (G-down) and rising BP via PE (G-up). Integrated gain was determined by plotting beat-to-beat R-R intervals against systolic BP. The mechanical and neural components were calculated from plotting diameter vs systolic BP, and R-R intervals vs carotid artery diameter, respectively. A novel analysis method based on linear mixed models (Atkinson et al., 2010) was employed to compare the integrated, mechanical and neural gains between 0700 and 1600h. An attenuated (P<0.05) baroreflex gain was found in the morning ([mean ±SE] G-up = 13.0 ± 0.6; G-down = 6.3 ± 0.4 ms/mm Hg) compared with the afternoon (G-up = 15.1 ± 0.6; G-down = 12.6 ± 0.4 ms/mm Hg). For rising pressures, the reduced integrated gain in the morning was explained by a smaller (P=0.004) mechanical gain (0.015 ± 0.001 mm/mm Hg) compared with the afternoon (0.018 ± 0.001 mm/mm Hg). However, the reduced falling pressure was explained by a diminished (P<0.0005) neural gain in the morning (256.0 ± 30.6 ms/mm) compared with the afternoon (494.9 ± 48.8 ms/mm). Our findings explicate the underlying mechanisms of diurnal variation in BP control. We suggest that the high prevalence of cardiovascular events in the morning is due to diminished mechanical transduction of pressure into arterial distension at this time. Physical activity and pharmacological interventions targeting the enhancement of barosensory vessel compliance and distensibility may improve the mechanical component of the cardiac baroreflex response, thus reducing the risk of cardiovascular events following waking. Atkinson et al. (2010). Clin Physiol Funct Imaging 31, 80-82 Kool et al. (1991). J Hypertens Suppl 9, 108-109 La Rovere MT et al. (1988). Circulation 78, 816-824 This study was funded by Liverpool John Moores University’s Institute for Health Research.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
The effect of a non-obesogenic high-fat diet on excitation-contracture coupling in cardiomyocytes

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High-fat diet can trigger cardiomyopathies that are normally associated with obesity-induced co-morbidities (e.g. diabetes, hypertension). However, high-fat diet can also directly trigger cardiac changes without obesity, by altering cardiac metabolism/function. The aim of this work was to investigate the effects of high-fat diet, pre-obesity and associated co-morbidities, on cardiomyocyte contractility and calcium cycling. Male C57BL/6 mice were fed either a normal rodent diet (13% kcal from fat) or a high-fat diet (45% kcal from fat and 0.15% cholesterol) for 24 weeks. Cardiomyocytes were isolated using a perfusion protocol which contained collagenase type-1 and protease to digest the heart. The isolated cells were then perfused with a HEPES buffer (pH 7.4). Ca$^{2+}$ transients were measured using the ratiometric Fura-2 AM dye and the contraction was monitored using an edge tracking device. Data are presented as mean±SEM and analysed using ANOVA with Fisher’s PLSD post-hoc test.

Feeding male C57BL/6 mice a high-fat diet for 24 weeks elevated blood cholesterol (148±10 to 214±11mg/dl, n=18-25/group) and triglycerides (79±16 to 187±14mg/dl, n=18-25/group) but had little effect on body weight (31.72±0.31 vs. 32.37±0.43g, n=50/group) and did not change blood glucose (135±25 vs. 136±7mg/dl, n=18-25/group).

Percentage of twitch contraction (stimulated at 0.2Hz) increased from 7.2±0.3% in the normal diet to 12.7±0.3% in the high-fat diet, P<0.0001 (n=23-41 cells/group from 4-6 hearts/group). However, the amplitude of the Ca$^{2+}$ transient was not different between the normal and high-fat diets (139±3% vs. 136±5%, P>0.05, n=19-32 cells/group from 2-4 hearts/group). This effect was also seen at different stimulation frequencies (0.5, 1.0 and 2.0Hz). In each frequency the time to peak contraction was significantly quicker in the high-fat group compared to the normal diet group, P<0.05. This did not correspond to a faster time to peak of the Ca$^{2+}$ transient in high-fat diet compared to normal diet, P>0.05, except at 0.2Hz, P<0.05.

These data show that isolated cardiomyocytes from mice fed a high-fat diet had a decreased time to peak shortening and increased amplitude of shortening during contraction compared to normal diet cardiomyocytes, which did not correlate with a faster time to peak and greater amplitude of the Ca$^{2+}$ transient respectively. This contractility modification by high-fat diet could be attributed to increased myocardial sensitivity to Ca$^{2+}$, or a change in the viscoelastic properties of the cardiomyocytes. Modifications to contractility and Ca$^{2+}$ handling could have implications in the increased vulnerability to ischaemia-reperfusion in the high-fat group, either through Ca$^{2+}$ overload and mitochondrial permeability transition pore opening or changes in ATP usage.

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Altered GABA$_A$ and NMDA receptor expression in the hypothalamic paraventricular nucleus of hypertensive and pregnant rats

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The autonomic nervous system plays an essential role in regulation of the cardiovascular system, and over activation of the sympathetic branch has been shown to be characteristic of many cardiovascular diseases, including hypertension. Interestingly however, sympathoexcitation is also required to maintain cardiovascular function vital for foetal development during pregnancy.

The paraventricular nucleus of the hypothalamus (PVN) is an important brain site for integration of cardiovascular inputs and a known site for autonomic regulation. Studies have shown that GABA elicits inhibitory effects within the PVN through the GABA$_A$ receptor, whereas glutamate has an excitatory effect through the NMDA receptor. Under normal physiological conditions, GABAergic inhibition suppresses autonomic outflow from this nuclei. However, electrophysiological evidence has shown that in hypertension, glutamatergic activity is increased and GABAergic inhibition is attenuated.

In this study, we aimed to investigate the composition of GABA$_A$ and NMDA receptors in the PVN in the hypertensive and pregnant rat to determine the contribution these receptors may make to altered sympathetic nerve activity.

All experiments were performed in accordance with the Animals (Scientific Procedures) Act, 1986. Animals (female Wistar rat (n=4), female spontaneously hypertensive rat (SHR; 14wk, n=4) and pregnant Wistar rat (E19, n=4)) were euthanized with sodium pentobarbital (60mg/kg intracardial), brains were extracted and frozen in isopentane (-20°C). The PVN was located using neuroanatomical landmarks and two 600µm slices were taken. Bilateral punches were then taken and subjected to quantitative immunoblotting for the GluN1 and GluN2A subunits of the NMDA receptor and the α1 subunit of the GABA$_A$ receptor. Results were normalised against β-actin and analysed using ImageJ.

Expression of the GluN1 and GluN2A subunit of the NMDA receptor and GABA$_A$α1 subunit was found throughout the rostrocaudal extent of the PVN in all models. In the pregnant animal, a significant decrease (p<0.05) in expression of the GABA$_A$α1 subunit was observed, but no significant changes were observed in expression of the NMDA receptor subunits.

In the SHR, expression of the GluN2A subunit was significant increased (p<0.05), while, expression of the GABA$_A$α1 subunit was significantly decreased (p<0.05). This is the first study to examine the subunit conformation of the GABA$_A$ and NMDA receptor in the PVN of hypertensive and pregnant animals. We have shown that these animals show differential changes in their inhibitory and/or excitatory receptor subunit conformations. These changes may contribute to the sympathoexcitation characteristic of these states.


Funded by BBSRC
Chronic electrical stimulation of the ventral lateral periaqueductal grey (vlPAG) evokes a persistent and substantial hypotensive response in spontaneously hypertensive (SH) but not normotensive rats

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Deep brain stimulation of the vlPAG is used for the clinical treatment of chronic neuropathic pain in man. It also produces sustained reductions in arterial pressure (AP) in hypertensive patients (Patel et al. Neurology, 2011). We have developed an implantable electrical stimulation system and applied it to the vlPAG of conscious, freely moving rats. AP and heart rate (HR) responses were measured via telemetry to explore potential central mechanisms.

Devices were implanted under ketamine and dormitor anaesthesia (60mg.kg-1/250ug.kg-1 intraperitoneal injection). Quiescent rats were stimulated for 60 min at a series of frequencies (10-40 Hz) with >30 min recovery between steps. SH rats (mean baseline AP 139±10mmHg; n=4) showed sustained and frequency-dependent AP reductions (12±2 vs 27±4mmHg; 10 vs 30 Hz, peak response; p<0.05). In contrast, in Wistar rats (mean baseline AP 101±9mmHg; n=2) smaller depressor responses were evoked (3±2mmHg vs 6±2mmHg; 10 vs 30Hz, peak response) which tended to return towards baseline levels after 20-30mins stimulation. Corresponding decreases in the low frequency (LF) power of systolic blood pressure were observed (SH 8.8±2.3 vs 1.9±0.5mmHg2; Wistar 1.4±0.5 vs 1.1±0.4mmHg2; baseline vs 30Hz), indicating that sympathetic outflow may be reduced.

In SH rats the depressor response was maintained throughout 7 days of continuous 20Hz vlPAG stimulation (135mmHg to 121mmHg; baseline vs day 6; n=3) and associated with a reduction in the low frequency power of systolic blood pressure (4.8±2 vs 1.4±0.2mmHg2; baseline vs day 6). In Wistar rats the reduction in blood pressure was smaller (99 vs 95mmHg) and no change in LF power observed (0.5 vs 0.4mmHg2).

Thus, the vlPAG of the SH rat is a more effective depressor site than in Wistar rats, and this may be due to a more pronounced suppression of the sympathetic nervous system. Our current studies are now determining the mechanisms for this.

We are grateful for the support of the Royal Society and the NIH.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
The acute inotropic and electrophysiological response during cardiac contractility modulation is mediated by local post-synaptic transmission in the isolated innervated rabbit heart

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The cardiac ventricle is richly innervated by both efferent and afferent sympathetic fibres. The latter relays sensory information from the myocardium to the intracardiac and intrathoracic ganglia, spinal cord and the brain for integration and is known to be involved in the regulation of cardiac function. We have previously demonstrated in isolated hearts that application of electrical signals to the rabbit ventricle during the absolute refractory period enhances inotropy by stimulating the release of noradrenaline - so-called cardiac contractility modulation (CCM) (1). The aim of the present study was to investigate the potential role of the intracardiac, intrathoracic ganglia and afferent sympathetic nerves in the inotropic and electrophysiological responses seen during CCM. Experiments were conducted in the isolated innervated New Zealand white rabbit hearts (n=4). Following pre-sedation (ketamine (10mg/kg), medetomidine hydrochloride (0.2mg/kg) and butorphanol (0.05mg/kg) (i.m.)) general anaesthesia was established with propofol (1%w/v ad libitum, i.v.) during which vessels were ligated and cervico-thoracic tissues isolated to give the innervated heart preparation. Animals were euthanised with pentobarbitone overdose (160mg/kg, i.v.). The resulting ex vivo preparation was perfused via the descending aorta with constant flow. Left ventricular (LV) performance was measured iso-volumetrically with a fluid filled balloon. Biphasic square wave electrical pulses (amplitude=15mA, duration=20ms) were applied to the LV using wire electrodes and timed to coincide with the absolute refractory period measured from locally recorded monophasic action potentials. Local action potential duration (APD) was calculated both immediately before CCM and immediately after the cessation of stimulation. The inotropic and electrophysiological effects of CCM were assessed before and during perfusion of the nicotinic ganglionic transmission inhibitor hexamethonium (HX, 0.5mM) and following removal of the spinal cord. Data (means±SEM) were compared using paired Students T-Tests. CCM enhanced LV pressure (LVP, Fig1A), rate of pressure development (Fig 1B) and promoted the shortening of local APD (Fig1C). The effect of CCM was comparable before and after removal of the spinal cord (data not shown). Our data provides functional evidence that CCM does not engage the intracardiac or intrathoracic ganglion and that afferent nerve transmission is not required to modulate the acute ventricular response during CCM. These data support the notion that the acute inotropic and electrophysiological effects of CCM are mediated through local post-synaptic noradrenaline release.

**Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.**

PC21

The acute inotropic and electrophysiological responses to CCM. BL=Baseline, CCM=Cardiac Contractility Modulation, LVP=Left ventricular pressure, dp/dtmax=maximal rate of change of pressure, MAPD= Mono-phasic action potential duration. *P<0.05, **P<0.01, ***P<0.001.

Winter J et al. (2010). Europace 12(suppl 2), ii4-ii7

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PC22

Peripheral chemoreceptor inputs contribute to the development of high blood pressure in spontaneously hypertensive rats

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The peripheral chemoreceptor (PC) reflex is enhanced in subjects with hypertension (Trzebski et al. 1982). In the spontaneously hypertensive rat (SHR), carotid body glomus cells from juvenile SHR show hypersensitivity to acidosis even before the onset of hypertension (Tan et al. 2010). Furthermore, prehypertensive SHRs (PH-SHR) display enhanced sympathoexcitatory responses to PC stimulation (Tan et al. 2010). We hypothesized that heightened PC activity in PH-SHR contributes to the development of hypertension in this rodent model of neurogenic hypertension in later life and that this is caused by pro-inflammatory cytokines resident in the carotid body of the PH-SHR. All procedures conformed to the UK Home Office guidelines on animals (Scientific Procedures) Act 1986 and were approved by the University of Bristol’s Animal Ethic Committee. In 4-5 week PH-SHR (n=7) and age- and sex-matched Wistar rats (n=7) the carotid body was ablated under general anaesthesia (ketamine 60 mg.kg-1 with medetomidine 0.25 mg.kg-1 i.p.); depth of anaesthesia was assessed by the absence of a withdrawal response to a noxious pinch to the tail. Sham operated rats were also prepared (PH-SHR and WKY; 4-5 weeks old; n=7 each). One day after the surgery all animals with carotid body ablation failed to respond to hypoxia (10% O2) confirming full excision. Five days prior to measurement of arterial pressure (AP), when rats were 12 weeks old, all animals (carotid ablated and sham) were re-anaesthetised (Halothane, 4% in oxygen) and a catheter was inserted into the femoral artery to measure arterial pressure chronically from conscious, freely moving animals.

Adult carotid body excised SHRs (12 weeks) showed lower mean AP than sham operated SHRs (121±3 vs 145±10 mmHg, mean ± SEM, ANOVA, SNK, P<0.05). The mean AP of SHR with carotid body ablation failed to respond to hypoxia (10% O2) confirming full excision. Five days prior to measurement of arterial pressure (AP), when rats were 12 weeks old, all animals (carotid ablated and sham) were re-anaesthetised (Halothane, 3% in oxygen) and a catheter was inserted into the femoral artery to measure arterial pressure chronically from conscious, freely moving animals.

Adult carotid body excised SHRs (12 weeks) showed lower mean AP than sham operated SHRs (121±3 vs 145±10 mmHg, mean ± SEM, ANOVA, SNK, P<0.05). The mean AP of SHR with carotid body ablation remained significantly higher than both Wistar control groups (SHAM 95±3 mmHg, carotid body excised 101±3 mmHg, P<0.05). Immunohistochemical analysis of carotid bodies from sham SHRs revealed strong expression of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), IL-1β receptor I and TNF-α receptor II in tyrosine hydroxilase-positive glomus cells type 1.
In conclusion, carotid body excision in juvenile PH-SHR reduces the degree of hypertension in later adult life. We suggest that PC hypersensitivity induced by chronic inflammation contributes to the development of high sympathetic drive and concomitant hypertension in the SHR.


British Heart Foundation, NIH (1R01HL-076803). JFRP was in receipt of a Royal Society Wolfson Research Merit Award.

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**PC23**

**Post-cardiopulmonary bypass surgery acute kidney injury is associated with gene expression changes in a porcine model**

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Acute kidney injury (AKI) is one of the most serious complications post-cardiac surgery. The pathogenesis is poorly understood. Cardiopulmonary bypass (CPB) is a major contributor to AKI. We aimed to investigate gene expression changes related to post-cardiopulmonary bypass (CPB) AKI in a large animal model with greater homology to cardiac surgery patients.

Transcriptomic analyses were performed on kidney medulla tissues obtained from pigs undergoing CPB or Sham animals. Microarray results were validated with real-time polymerase chain reaction and gene products were estimated by semi-quantitative Western blotting.

Of the transcripts examined, 66 were identified as differentially expressed in CPB versus Sham pig’s kidney samples (t-Test P<0.05; 1.6 fold), with 19 (29%) up-regulated and 47 (71%) down-regulated. Only 5 and 18 out of the upregulated and downregulated transcripts respectively were annotated genes. The rest were ESTs. The regulated genes clustered into three classes Immune response, Cell adhesion/extracellular matrix and metabolic process. The upregulated genes included F5, SLC16A3 and CKMT2 whereas the downregulated genes included GST, CPE, MMP7 and SELL. Collectively, these changes indicate genetic remodelling of the kidney’s medulla in response to CPB.

This work was funded by the British Heart Foundation and the NIHR Bristol BRU in Cardiovascular Medicine.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC24**

**Altered cardiac fibroblast proliferation during cardiac hypertrophy is modulated by calcium/calmodulin-dependent protein kinase IIb**

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Left ventricular hypertrophy (LVH) is an adaptive response to myocardial injury. Adaptations of the hypertrophic heart include (i) cardiac myocyte enlargement and calcium dysregulation, (ii) fibrosis due to collagen deposition by cardiac fibroblasts (CFs) and (iii) chronic inflammation. Calcium/calmodulin-dependent protein kinase IIb (CaMKIIb) has been highlighted as a key modulator of hypertrophic cardiac handling however, little is known of its potential involvement in cardiac fibrosis or inflammation. Here, we examine the role of CaMKII in CF proliferation, an event that when uncontrolled, underlies development of fibrosis. We have developed and characterised a minimally invasive transverse aortic banding (MTAB) mouse model of LVH. Mice (C57, 25-30g) were anaesthetised with 3% Isoflurane in oxygen, maintained with 1.5% Isoflurane in oxygen and were given analgesic (60μg/kg Buprenorphine) intramuscularly. Heart weight:body weight ratios are significantly increased in MTAB compared to sham animals (6.4±0.5, n=12 cf. 5.1±0.18, n=11, p<0.0001). LV contractility (% fractional shortening) is significantly depressed (33.8±1.6 cf. 41±2.8, p=0.03, in MTAB (n=12) versus sham (n=10)) and myocardial fibrosis, assessed by picrosirius red staining of collagen, is significantly increased (5.9±1.6 cf. 0.55±0.2 % area of staining in field measured), p=0.03, in MTAB (n=6) versus sham (n=4)). CaMKIIb protein expression, assessed in parallel in cardiac homogenates (ratio to GAPDH), is significantly increased (0.77±0.14 cf. 0.35±0.03, p=0.03, in MTAB (n=9) versus sham (n=6)). Using CFs isolated from MTAB and sham animals, we have assessed whether (i) proliferation rates are altered following hypertrophy and (ii) whether CaMKIIb may regulate CF proliferation. In response to Angiotensin II (1μM), CF proliferation (assessed by mean cell count from 10 random fields of view) increased over 72h with a significant difference in cell numbers between MTAB and sham cells evident after 48h (59.6±2.5 cf. 41.8±2.3, respectively, p=0.006, n=3). When cells were pre-treated for 1h with 5μM AIP (a specific inhibitor of CaMKII), proliferation was significantly decreased at 24h and this decrease was maintained at 48h and 72h in both sham (72h; 50.9±3.3 (untreated) cf. 29.8±0.6 (AIP-treated), respectively) and MTAB cells (72h; 71.0±7.2 (untreated) cf. 37.5±1.5 (AIP treated), respectively). This represented a 42% decrease in proliferation in sham cells and a 48% decrease in proliferation in MTAB cells following AIP treatment (n=3). Importantly, in cells isolated from both groups, AIP pre-treatment had no obvious effect on cell number at time zero (AIP-treated v’s untreated cells, sham p=0.270 and MTAB p=0.105). These results highlight a potential role for CaMKIIb in regulating CF proliferation during both normal and hypertrophic growth.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
IP3R expression is increased in a minimally invasive surgical mouse model of cardiac hypertrophy

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Cardiac hypertrophy is an adaptation to cardiac insult and can be used as an accurate predictor for development of heart failure. Abnormal calcium handling is a key process contributing to compromised cardiac function during cardiac hypertrophy. It is unclear whether the inositol-1,4,5-trisphosphate receptor type 2 (IP3R2) calcium release channel plays a role in the cardiac dysfunction that accompanies hypertrophy. Using a minimally invasive surgical model of transverse aortic banding (MTAB), IP3R2 protein expression was assessed in ventricular tissue from sham-operated and MTAB animals. Mice (20-25g, adult C57) were anaesthetised with 3% isoflurane and maintained on 1% isoflurane, both in 100% oxygen. Aortic arch ligation was performed without entering the pleural space (minimally invasive). A 27G needle was used to control the tightness of the band. Sham animals underwent the same procedure except the arch was not ligated. Contractile function was measured by echocardiography 14 days and 28 days after surgery. Left ventricular (LV) wall thickness (anterior wall and posterior wall thickness) and internal dimensions during diastole and systole (LVDD/LVESD) were measured. LV fractional shortening, relative wall thickness and LV mass were calculated from short-axis view M-mode measurements. After hemodynamic recordings were completed, the hearts were rapidly excised following pentobarbital sodium overdose (intraperitoneal, 10 μl/g). Ventricular tissue was homogenized for quantitative western blotting or processed for histology. MTAB animals developed severe hypertrophy by week 4 as assessed by a significant increase in heart weight to body weight ratio (5.25 ± 0.33 v's 8.83 ± 0.58, p < 0.001, sham, n=7 v's MTAB, n=11 respectively) with a 1.7-fold increase in LV mass in the mouse heart. Fractional shortening was decreased by 50.5% (49.00 ± 3.34 v's 24.78 ± 2.44, p = 0.001, sham, n=6 v's MTAB, n=9 respectively) by week 4. Evidence for fibrosis was indicated by significantly increased collagen deposition as assessed by pico-sirus red staining (0.55 ± 0.2 v's 5.9 ± 1.6, p < 0.03, sham, n=4 v's MTAB, n=6 respectively). IP3R2 protein expression (normalised to GAPDH) increased by approximately 2.9 fold in hearts from MTAB mice in comparison to sham (IP3R2:GAPDH ratio: 0.378 ± 0.06 v's 1.303 ± 0.03, p = 0.002, sham, n=7 v's MTAB, n=11 respectively). There was a significant correlation between the gradient of slopes of IP3R2:GAPDH ratio and fractional shortening, where a decreased fractional shortening correlated with an increased IP3R2 expression (p < 0.05, Spearman r = -0.73, n=13). In summary, there is a significant elevation in ventricular IP3R2 protein expression following pressure-overload--induced cardiac hypertrophy. A strong correlation exists between increasing IP3R2 protein levels and progression of cardiac dysfunction. Further investigations will explore the mechanistic contribution of altered channel expression to cardiac function.

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Atrial IP3 receptor expression is increased in patients with left ventricular systolic dysfunction

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Introduction: The arrhythmogenic mechanisms of atrial fibrillation (AF) are poorly understood, but likely involve a contribution from a pathological remodelling of atrial intracellular Ca2+-handling proteins. One candidate, highly expressed in human atrium, is the inositol 1,4,5-trisphosphate receptor (IP3R). It is unknown whether heart failure or left ventricular systolic dysfunction (LVSD), major causes of AF and atrial remodelling, also affect the expression of human atrial IP3R.

Aims: To compare atrial IP3R protein expression levels between patients with and without LVSD, and to investigate correlations between IP3R expression and the LV ejection fraction (LVEF). Methods: Right atrial appendage tissues were obtained from 20 consenting patients with or without LVSD, who were in sinus rhythm and undergoing cardiac surgery. Tissues were homogenised and stored at -80 °C. After randomisation and blinding of homogenates, IP3R2 levels were quantified by western blotting using anti-IP3R type 2 antiserum and GAPDH as an internal control. To ensure that the protein load of homogenates was in the linear range of the densitometer, a pre-determined range of protein concentrations was tested, and protein loads of 6, 9 and 12 μg were selected. IP3R2:GAPDH ratios were obtained at each protein load, and the slope of the three IP3R2:GAPDH ratios was calculated for each sample. The mid-range (9 μg) load was also evaluated alone for comparison. Results: Patients with moderate or severe LVSD had a significantly higher atrial IP3R2:GAPDH ratio (9 μg protein load) than those with no LVSD (3.97 ± 0.47, n=10 patients versus 2.54 ± 0.13, n=10 patients; p<0.05, unpaired 2-tailed Student’s t-test with Welch’s correction). There was a correspondingly higher gradient of the slope of IP3R2:GAPDH ratios (6-12 μg protein load) in patients with moderate or severe LVSD than in those with no LVSD (0.33 ± 0.02 versus 0.20 ± 0.01, p<0.001). Furthermore, in the patient cohort as a whole, there was a significant correlation between the patients’ LVEF value (where available) and the IP3R2:GAPDH ratio (9 μg protein load), such that a decreasing LVEF correlated with increasing expression of IP3R2 (p<0.05; Spearman r = -0.70, n=9 patients). Conclusions: In patients in sinus rhythm and undergoing cardiac surgery, an increased expression of the atrial Ca2+-handling protein IP3R2 was independently associated with worsening LV systolic function. Such increased IP3 receptor expression might have the potential to contribute to the predisposition to AF in patients with LVSD or heart failure.

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Investigation of cardiovascular parameters as indicators of sympathetic activity in lean and hypermetabolic Gnasxl knock-out mice

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Obesity is a growing problem worldwide; anti-obesity drugs are being developed through the use of genetically modified mouse models. We are investigating the role of the autonomic nervous system in a lean knock-out (KO) mouse model, the Gnasxl KO. Gnasxl encodes XLαs, which is an extra-large variant of the G-protein subunit, Gαs, a signalling protein involved in cAMP production. Adult mice lacking XLαs are lean and hypermetabolic, showing increased energy expenditure and lipolysis (1). They also exhibit increased urinary catecholamine excretion, suggesting the phenotype may be caused by increased sympathetic tone (1). Our aim is to analyse cardiovascular parameters to determine the extent of sympathetic nervous system (SNS) involvement in the KO phenotype. Other data from our lab suggest a central cause, due to the restricted expression pattern of XLαs (2), which includes regions of the hypothalamus and medulla that are critical for sympathetic control. We investigated three indicators of SNS activity in adult male Gnasxl KO mice: blood pressure, body temperature and heart rate. Results are given as mean ±SEM. Blood pressure was measured by non-invasive tail plethysmography in urethane-anaesthetised mice (1.3-2.1 mg/g ip), and by arterial cannulation in dose-matched urethane-chloralose anaesthetised mice (urethane 1.4-2.2 mg/g ip; chloralose 7.0-11.0 μg/g ip). Using tail plethysmography, mean blood pressure was increased in Gnasxl knock-outs (KO) (79.4 ±6.6 mmHg, n=9) compared to wild-types (WT) (66.1 ±3.2 mmHg, n=8), p<0.05 by ANOVA. This was also seen using arterial cannulation, with increased blood pressure in KO (64.6 ±4.9 mmHg, n = 10) compared to WT (48.5 ±4.5 mmHg, n=9), p<0.05 by t-test. In urethane-chloralose anaesthetised mice (urethane 1.2-2.4 mg/g ip; chloralose 6.0-12.0 μg/g ip) core body temperature, measured by rectal probe, was elevated in KO (38.0 ±0.16 °C, n=13) compared to WT (36.6 ±0.30 °C, n=12), p<0.001 by t-test. ECG was recorded in conscious, freely moving mice by telemetry; transmitters were implanted under inhalation anaesthesia (isoflurane), and ECG was recorded after a recovery period. Continuous ECG data were digitised (DSI, UK) and processed with custom and PhysioNet software (3). Mean heart rate measured over a 24 hour period was elevated in KO (626 ±40 bpm, n=3) compared to WT (561 ±28 bpm, n=3), p<0.05 by paired t-test. Together, these results suggest that Gnasxl KO mice have increased sympathetic stimulation of the cardiovascular system, consistent with a basal increase in sympathetic tone. In future experiments, we will investigate the activity of neuropeptides and antagonists on the SNS in the Gnasxl KO.

Xie T., et al. (2006), J. Biol. Chem. 281: 18989-18999

This work was supported by a “Capacity Building Award in Integrative Mammalian Biology”, a BBiSRC “Special Skills” grant and by the MRC.
CO on the recombinant human cardiac Na\textsubscript{v}1.5 channel stably expressed in HEK293 cells\textsuperscript{2}. The CO-releasing molecule CORM-2 inhibited the peak inward current amplitude (I\textsubscript{peak,Na}) without affecting the late current (I\textsubscript{late,Na}) in a voltage-independent manner (I\textsubscript{CORM}= 1.05 μM). Exposure to 3 μM CORM-2 elicited 71 ± 4.7% decrease of basal I\textsubscript{peak,Na} (mean ± S.E.M., n = 21 cells) whilst 3 μM iCORM (the inactive form of the donor) only reduced currents by 4.5 ± 5.2% (n = 9). Inhibition mediated by 3 μM CORM-2 was unaffected by general or mitochondria-targeted antioxidants (0.5 mM ascorbic acid, 3 mM GSH, 0.4 mM Trolox, 0.1 mM MnTMPyP or 0.3 μM Mitochondria Q) and was also unaffected by blockers of mitochondrial electron transport (2 μM rotenone, 1 μM stigmatellin, or 3 μM antimycin A). Inhibitors of protein kinase G (50 μM Rp-8-Br-PET-cGMPS), CalMKII (1 μM KN-93), and p38MAP kinase (10 μM SB203580) were also unable to alter the effects of CO on I\textsubscript{peak,Na}. In contrast, CO-mediated inhibition was dramatically reduced by the nitric oxide synthase (NOS) inhibitor L-NAME (1 mM, 1 h incubation) (14.3 ± 9.9% inhibition, n = 8, p < 0.01 compared to % inhibition induced by 3 μM CORM-2 alone). CO raised NO levels in HEK293 cells, as monitored using the nitric oxide (NO)-sensitive fluorescent dye DAF-2. This NO production was blocked by L-NAME, but NO donors (0.2 mM SNAP and 0.1 mM SIN-1) did not mimic CO effects on I\textsubscript{peak,Na}. However, CO-mediated inhibition was reduced or prevented by the reducing agents dithiothreitol (1 mM; 43.7 ± 7.3% inhibition, n = 11, p = 0.01) and L-cysteine (100 μM; 48.6 ± 9.0% inhibition, n = 7, p < 0.05).

Our results indicate that the inhibition of I\textsubscript{peak,Na} by CO are largely mediated by a rise of NO produced by localized NOS, and suggest that the channel redox status might be an important determining factor in this effect of CO.

Supported by the British Heart Foundation.

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**PC30**

**Macrophage apoptosis and loss of motility is induced by asymmetric dimethylarginine**

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Asymmetrically methylated forms of arginine (asymmetric dimethylarginine, (ADMA) and L-N-monomethylarginine, (L-NMMA)) are competitive inhibitors of all three isoforms of nitric oxide synthase (NOS), the enzyme responsible for the conversion of L-arginine to NO \textsuperscript{1}. Nitric oxide (NO) is a critical signalling molecule that amongst other effects can deplete macrophages in atherosclerotic plaques \textsuperscript{2}. Additionally, the ADMA/NOS pathway is a key regulator of endothelial cell motility \textsuperscript{3}, although its role in monocyte and macrophage motility remains unclear.

U937 cells, a human leukemic monocyte lymphoma cell line, were differentiated into macrophages using 20-50ng/ml phorbol ester (PMA). To test cell proliferation Interferon-γ (10U/mol) and ADMA (10μM, 50μM, and 100μM) were added either singly or in combination (24 h before) and incubated with the tetrazolium salt [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] for 3 h. The absorbance at 570nm was measured. Nitrite levels were measured by incubating the supernatant of treated cells with Griess reagents [sulfanilamide and N-1-naphthylethenediamine dihydrochloride] for 30 min and measuring the absorbance at 540nm. Here we show that in U937 differentiated monocytes, cell proliferation decreases significantly (P<0.05; n=3) with ADMA (50μM, 100μM) concomitantly with decreased NO production, suggesting that ADMA may induce a reduction in NO release or NOS activity and cell proliferation as a result of cell apoptosis.

Subsequent migration of differentiated monocytes was also digitally recorded by microscopy with a time interval of 10 min for a period of 12 h. ADMA (1μM, 10μM and 100μM) was shown to significantly reduce motility and directional travel dose dependently by approximately 0.3 μm/min (P<0.05; n=6). Symmetric dimethylarginine (SDMA), which does not inhibit NOS, had variable dose dependant effects and reduced motility and directional travel at pathophysiological concentrations (100μM). Subsequent immunostaining of f-actin using an Alexa Fluor 488® phalloidin demonstrated an increase in stress fibre and focal adhesion formation following incubation with ADMA (100 μM).

This implicates ADMA as a critical regulator of monocyte/macrophage motility and highlights the possible therapeutic benefits of pharmacological tools to promote NO production and/or increase ADMA removal from the body.


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**PC31**

**Electrophysiological properties and calcium handling in the atrium in mice with cardiac specific deletion of plasma membrane calcium ATPase isoforms 1 (PMCA1)**

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The regulation of intracellular Ca\textsubscript{2+} plays a crucial role in maintaining normal cardiac function. It is generally thought that plasma membrane calcium ATPase (PMCA) plays a relative modest role in extrusion of Ca\textsubscript{2+} from the cytosol comparing with SERCA and Na-Ca exchanger (NCX) (Brini, 2009). However, recent studies have shown that both PMCA 1 and 4 play critical roles for modulating cardiac contractility, hypertrophy, intracellular signalling pathways, calcium homeostasis and vascular tone (Brini & Carafoli, 2009; Cartwright et al., 2009). The role of PMCA1 in atrium electrical function and intracellular Ca\textsubscript{2+} regulation, however, has not been previous explored. In the present study, we characterized the electrophysiological properties and calcium handling in the atrium in mice with
cardiac specific deletion of plasma membrane calcium ATPase isoforms 1 (PMCA1cko).

Mice at 3-4 months old were used for the study. The expression and cellular distribution of PMCA proteins were characterized by Western blotting and immunostaining. Conventional ex vivo isolated Langendorff-perfused heart preparations were used for ECG, monophasic action potential (MAP) and multi-electrode array recordings of extracellular potentials (ECPs) in both control and PMCA1cko mice. The electrophysiological properties and intracellular calcium handling were investigated by isolated atrial cardiac myocytes with whole cell patch clamping.

Immunostaining indicated PMCA1 localized at cell membrane and caveolae in control mice. Both Western blotting and immunostaining demonstrated an efficient deletion of PMCA1 protein in atrial myocytes in PMCA1cko mice. PMCA1cko hearts were susceptible to atrial arrhythmias including atrial tachycardia (AT) and atrial fibrillation when given electrical program stimulation. Out of 6 PMCA1cko mice examined developed AT/AF compared to 0 out of 5 control mice examined.

Single cell study indicates the L-type Ca2+ channel current was reduced approximately 20% in PMCA1cko mice compare with control mice (control n=23, PMCA1cko n=9). There was no significant difference in SR content and current amplitude of NCX (control n=11, PMCA1cko n=15), however, the NCX current decay was prolonged by approximately 40%. Increase external Ca2+ concentration from 1 mM to 2 mM, action potential alternans, early after depolarisation (EAD), delayed after depolarisation (DAD) as well as spontaneous Ca2+ wave were frequently seen in PMCA1cko myocytes. In conclusion, PMCA1 may play a role in maintaining atrial electrical function and intracellular Ca2+ homeostasis, particularly, under stress condition.


This work is supported by The Wellcome Trust and The British Heart Foundation.

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**PC32**

The role of catecholamines in the augmented chemosensitivity of heart failure

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Augmented chemosensitivity has been observed in chronic heart failure (CHF: Sun et al, 1999) and its subsequent effects, including sympathetic hyperactivity have been shown to be instrumental in the progression of the disease (Ponikowski et al, 2001). We have investigated the role of plasma catecholamines in the chemosensitivity in CHF.

CHF was induced in male wistar rats (200-300g) following administration of isoproterenol by osmotic mini-pump which was implanted under inhalation anaesthesia (isoflurane 3.5% in oxygen) with subsequent pain relief administered as required (Temgesic, s.c. administration) (10 days - 4mg/kg/day s.c., Alzet mini pump). At 10 days, animals were instrumented, under anaesthesia (urethane, 1.4g/kg i.p.), to measure ventilation and arterial blood pressure. Acute experiments were performed on CHF (n=6) and normal rats (n=9) to assess the cardiovascular and respiratory responses to acute hypoxia (incremental reduction of inspired gases to 10% oxygen, balance nitrogen) under 3 conditions: Control (no infusion); supplemental noradrenaline infusion (10µg/kg/min) and after administration of the beta adrenoceptor antagonist, propranolol (1mg/kg). Exponential functions with offset were fitted to all ventilatory response curves. Values are means ± S.E.M. and compared by ANOVA with significance as p<0.05.

Infusion of NA to normal animals augmented ventilation at all concentrations of inspired oxygen, but particularly in hypoxia (112.3±6.1 vs. 78.4±6.6 ml/min at 100mmHg PaO2, P<0.05. 178.0±7.6 vs. 128.5±8.3 ml/min at 50mmHg PaO2, P<0.05, in the noradrenaline and control responses respectively). Subsequent administration of propranolol returned the NA augmented ventilation back to the control hypoxia response in normal animals. CHF rats had an elevated ventilatory response to hypoxia (156.3±9.5 ml/min at 50mmHg PaO2, P<0.05, compared to normal rats). Infusion of NA to CHF rats was without effect, indicating maximal adrenoreceptor activation has already occurred such that the elevation in ventilatory response to NA was significantly attenuated compared to the normal animals (0.9±8.5 vs. 49.6±7.6 ml/min in CHF and normal, respectively at 50mmHg PaO2, P<0.05). Propranolol attenuated the ventilatory response to a greater extent in the CHF rats than in normal rats, confirming a higher resting level of tonic catecholamine activity in CHF (43.7±7.5 vs. 21.1±5.9 ml/min at 50mmHg PaO2, P<0.05).

This may support the value of using beta-adrenoceptor antagonism in the management of CHF. Bilateral carotid sinus nerve section (CSNX) in normal rats (n=6) blunted the ventilatory response to NA by 20% (139.8±21.1 vs. 112.3±9.5 ml/min at 50mmHg PaO2, P<0.05, compared to normal rats). Infusion of NA to CHF rats was without effect, indicating maximal adrenoreceptor activation has already occurred such that the elevation in ventilatory response to NA was significantly attenuated compared to the normal animals (0.9±8.5 vs. 49.6±7.6 ml/min in CHF and normal, respectively at 50mmHg PaO2, P<0.05). Propranolol attenuated the ventilatory response to a greater extent in the CHF rats than in normal rats, confirming a higher resting level of tonic catecholamine activity in CHF (43.7±7.5 vs. 21.1±5.9 ml/min at 50mmHg PaO2, P<0.05). These results suggest that NA may act directly upon carotid chemoreceptors to increase chemosensitivity and hence augment ventilation in CHF that could be alleviated by beta-blocker.


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findings suggest that the product of the Wilms’ tumor gene, Wt1, whose expression is increased in hypoxia, has a role in neovascularisation of the ischemic heart. Here we demonstrate that the integral membrane protein 2A (Itm2a), which has been implicated in myogenic cell differentiation, is up-regulated in response to hypoxia and Wt1. Exposure of mice to 8% O2 for 6 hours (Wagner et al. 2003) caused a 2-fold increase of Itm2a mRNA in the hearts (Student’s t-test, P<0.01, n=5). Hearts were collected after mice had been killed. Incubation of the myoblast cell line C2C12 at 1% O2 for 24 hours caused an 8-fold increase of Itm2a mRNA compared to cells at 21% O2. A reporter construct carrying approximately 3 kb of the Itm2a promoter was stimulated 2.3-fold in transfected C2C12 cells grown at 1% O2 for 24 hours caused an 8-fold increase of Itm2a mRNA in the hearts (Student’s t-test, P<0.01, n=5). Hearts were reduced 4-fold in Wt1-deficient embryos compared to their normal littermates (Student’s t-test, P<0.05, n=5). In conclusion, stimulation of Itm2a expression in response to hypoxia and Wt1 may provide a novel regulatory mechanism in the heart.


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PC34

Slow desensitization of muscarinic K+ channel is associated with chronic vulnerability to atrial fibrillation

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Background — Cholinergic stimulation (CS) and pulmonary venoatrial junctions (PVJ) play a key role in atrial fibrillation (AF) that is correlated with ageing. In contrast, cardiac vagal control decreases with age, and effects of continuous CS fade with time. Yet, their relationship and mechanism are unclear.

Methods and Results — Extracellular potentials were recorded in the PVJ region and atria in rabbit heart perfused physiologically in vitro to study AF induced by ACh and/or electrical pacing. Whole cell current clamp and voltage clamp were employed to measure action potential (AP) and ion currents in single myocytes isolated from left atrium (LA) and PVJ. Single cell quantitative PCR was used to estimate the abundance of mRNAs for receptor and ion channels that were visualized and located by immunofluorescence. During 30 s application of 10 μM ACh, episodes of fibrillation wavelet (FW) were longer and peak FW was higher at the centre of PVJ (CPV) than in LA (CPV 23.5±5.1 vs. LA 8.9±2.7 s, n=9). The dominant frequency of ectopic activity (DFEA) was lower in LA and faded away with FW, whereas DFEA hid in noise at CPV after FW fadeaway. After 2 h ACh application and in the absence of ACh, long lasting FW was induced by burst pacing, and DFEA was higher at CPV than in LA (16.1±2.4 vs. 9.7±2.6 Hz, n=9). During 30 s ACh application, the effects of ACh on RP hyperpolarization and APD shortening and muscarinic K+ current (iK_ACH) in LA and PVJ myocytes declined (RP: LA 20.7±3.1 vs. PVJ 18.4±2.8%, n=15/9; APD: LA 15.7±2.1 vs. PVJ 11.4±1.2%, n=15/9; iK_ACH: LA 40.8±2.3 vs. PVJ 29.3±3.2%, n=38/76) as a result of fast desensitization to ACh, which were reversible following wash-off of ACh. After 2 h exposure to ACh, the effects of ACh on RP and APD and iK_ACH faded further (RP: LA 57.9±3.7 vs. PVJ 83.2±4.6%, n=11/7; APD: LA 31.3±5.1 vs. PVJ 46.8±3.4%, n=11/7; iK_ACH: LA 58.4±6.4 vs. PVJ 42.6±7.1%, n=22/33) as a result of slow desensitization, which were largely irreversible following ACh wash-off. The mean intensity of M2 receptor (M2R) labelling in the cell membrane was reduced or internalised (M2R: LA 82.8±6.7 vs. PVJ 71.3±5.1%, n=37/24). As compared with LA myocytes, in PVJ myocytes, iK1 and iK_ACH, and the abundances of Kir2.1, M2R and Kir3.1/3.4 mRNAs were lower, and decrease in the effects of ACh on RP and APD and iK_ACH was smaller during the fast desensitization, but generally larger during the slow desensitization.

Conclusions — The fast iK_ACH desensitization may reduce the effects of CS and prevent the prolongation of cholinergic AF, whereas the slow iK_ACH desensitization causes irreversible APD shortening and thus increase chronic vulnerability to AF even in the absence of CS. The PVJ region is more susceptible to CS.

This work was supported by British Heart Foundation & National Natural Science Foundation of China

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PC35

The effect of chronic catecholamine treatment upon vascular reactivity

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Poor ejection fraction in the failing heart is compensated for, in part, by augmented release of circulating catecholamines (Francis et al., 1988). The resulting profile of β-adrenergic receptor (β-AR) desensitisation and down-regulation has been well characterised in cardiac tissue (Bouvier et al., 1989), though the vascular effects remain poorly understood. In this present study, we explored the effects of chronic catecholamine exposure on vascular reactivity in the context of heart failure.

Male Wistar rats (200-300g) were treated with the non-selective β-adrenergic agonist, isoprenaline, infused continuously via osmotic mini-pump (Alzet: 4mg/kg/day, subcutaneous administration) for 10 days, leading to cardiac diminished performance. Pumps were implanted under inhalation anaesthesia (isoflurane 3.5% in oxygen) and subsequently, pain relief was administered as required (Temgesic, s.c. administration). Rats were humanely killed by cervical dislocation. Abdominal aorta was freshly-isolated and dissected free of connective tissue, and aortic rings suspended in a myograph and isometric tension estimated in response to agonist challenge (noradrenaline, 1μM). Basal and maximal tension and contractility were quantified with reference to constriction initiated to a high K+ (75mM) depolarisation. All data represent mean ± S.E.M, analysed with student’s t-test and ANOVA as appropriate.
Isoprenaline-treated animals showed an increase in the level of basal vascular tension (control, 13.29 ± 0.48mN; treated, 15.98 ± 0.44mN; P<0.05). In both control and treated groups, measures of IC50 demonstrated equal inhibition of noradrenergic contractile responses following α1-AR blockade with prazosin (control, 10-7.5 ± 0.34M; treated, 10-6.8 ± 0.39M; P=0.06), while α2-AR blockade with yohimbine failed to inhibit vasoconstriction in either group. Vasodilatory responses were unaltered by isoproterenol treatment, either for endothelium-dependent (acetylcholine, 1μM) or independent (sodium nitroprusside, 0.1μM) mechanisms, or by stimulation of the downstream signalling cascade in vascular smooth muscle (8-bromo-cyclic-GMP, 100μM).

In conclusion, chronic catecholamine exposure does not lead to α-AR desensitisation or endothelial dysfunction in the rat aorta. Despite this, the basal tone of the vessel may be increased; a deficit which could contribute to dysregulated blood flow distribution in heart failure.


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PC36

Vesicular release of ATP in brainstem astrocytes evoked by pH changes

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The mechanisms of central nervous chemosensory function underlying detection of blood and brain pH levels are not completely understood. We have recently demonstrated that a small decrease in extracellular pH from 7.4 to 7.2 induces rapid increase in [Ca2+] in astrocytes located in the central chemosensitive area located at the ventral medullary surface (VMS) (Gourine et al. 2010). These increases were largely mediated by release of ATP. Here we sought to identify the mechanism of ATP release triggered by acidification. All experiments were performed on Sprague-Dawley rats (UCL breeding colony, London, UK). Methods for tissue culturing and preparation of slices have been described in (Gourine 2010). All recordings were performed at 35-37°C in HBSS. Acidification-induced [Ca2+] responses in VMS astrocytes were not affected by either carbonoxolone (10 μM, blocker pannexin hemichannels) or lanthanum (La3+: 100 μM - a connexin hemichannel blocker that does not affect gap junctions). However, both brefeldine A and bafilomycin A - inhibitors of vesicular transport and vesicular H+ ATPase, effectively abolished [Ca2+] excitation of VMS astrocytes evoked by lowering external pH. Given that these Ca2+ responses are mediated by ATP (Gourine et al 2010), these observations indicate that acidification leads to release of ATP triggered by acidification. ATP released in this manner then propagates Ca2+ excitation across the astrocytic network to provide excitatory tone to the respiratory rhythm generator.


This work is funded by Welcome Trust and British Heart Foundation (PG/08/009/24411)

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC37

Inotropic Changes From Cardiac Contractility Modulation (CCM) Are Associated With Catecholamine Release And Activation Of The Slow Delayed Rectifying Potassium Current

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Cardiac contractility modulation (CCM) is being developed as a potential electrical treatment for heart failure, whereby an electrical current is applied to the ventricle during the absolute refractory period. However mechanistic information on this inotropic therapy is lacking. The aim of this study was to investigate the inotropic and electrophysiological effects of CCM in isolated rabbit hearts. Experiments were conducted in Langendorff perfused isolated rabbit hearts under conditions of constant flow (2.0-3.0 kg, n=8). Animals were euthanised by pentobarbitone overdose (160mg/kg, i.v.) following sedation with ketamine (10mg/kg), medetomidine hydrochloride (0.2mg/kg) and butorphanol (0.05mg/kg) (i.m.). Biphasic square wave pulses (Duration=20ms, Amplitude=20mA) were applied to the basal region of the left ventricle, timed to coincide with the absolute refractory period as measured from locally recorded monophasic action potentials (MAPs). Inotropic enhancement was assessed as the change in peak left ventricular pressure (LVP) from baseline values (steady state response). Basal (local) and apical (distal) MAP duration at 90% repolarisation (MAPD90) were measured at steady state before CCM and at the end of 120 s of CCM. Responses were
assessed during perfusion with metropolol (1.8μM, β1 adrenoceptor antagonist) and HMR1556 (500nM, inhibitor of the slow delayed rectifying potassium channel (IKs)). Norepinephrine content was measured by ELISA from coronary effluent collected during CCM. CCM produced a 5±1% increase in peak LVP and resulted in a shortening of local MAPD90 (-19±3%) but had no effect on on distal regions (0±1%). The reduction in local MAPs was abolished with metropolol (-15±3 [CCM] vs. 1±1% [CCM+metropolol], P<0.01) and was associated with norepinephrine accumulation in the coronary effluent (Mean=53±11 pg/ml). Metropolol also abolished the CCM induced inotropic response. Perfusion with HMR1556 resulted in a significant reduction of CCM mediated local MAPD90 shortening (-27±2 vs. [CCM] vs. -21±3% [CCM+HMR1556], P<0.05). Inotropic changes with CCM are associated with a shortening of left ventricular action potential duration, partly via IKs activation, and in a manner dependent upon β-adrenoceptor stimulation resulting from local catecholamine release in isolated rabbit hearts. These data may be of clinical importance in the further development of this mode of therapy in heart failure.

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PC38
Sildenafil alleviates bronchopulmonary dysplasia by up-regulating Hypoxia inducible factor-1α
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Bronchopulmonary dysplasia (BPD) is a major cause of morbidity in premature babies with birth weights less than 1 kg. Recently, sildenafil has been clinically tested to improve the pulmonary function in BPD patients. With respect to its pharmacological mode of action, sildenafil inhibits phosphodiesterase-5, leading to cGMP elevation. However, little is known about the mechanism by which sildenafil releases the BPD symptom. We here examined the mechanism in respect to the HIF-mediated adaptation to hyperoxic injury. All animal procedures were performed in accord with the Seoul National University Laboratory Animal Maintenance Manual. To induce BPD, neonatal rats were kept with foster rats for 7 days in a Plexiglas hyperoxic chamber containing 85% oxygen. While opening the chamber once a day for one hour, we rotated fosterers daily to keep nursing neonates, and injected (i.p.) sildenafil (0.1 mg/g per day) or vehicle (DMSO) into neonates. Experiments were divided into three groups; normoxia, hyperoxia, and hyperoxia+sildenafil. After being taken out of the chamber, neonates were kept in room air and injected daily with drugs for a further 7 days. After the treatments, lungs were excised from neonates, and subjected to histological study. The morphometric analyses were performed in H&E-stained lung sections. The alveolarization was measured by mean cord length and alveolar surface area. The alveolarization was severely impaired in neonatal rats exposed to hyperoxia, and sildenafil recovered the alveolarization in the BPD model. Furthermore, HIF-1α in the lungs of sildenafil-treated rats was expressed in a higher level than those of BPD rats. Immunoblotting of CD31 (vessel formation marker) and VEGF (an angiogenic factor regulated by HIF-1) revealed that sildenafil effectively promoted vascular formation. Mechanistically, cGMP was accumulated by sildenafil on account of interfering PDE5. Sildenafil stimulation activated HIF-1α and its down-stream genes at protein or mRNA levels in the human small airway epithelial cell line. We further confirmed that cGMP is a mediator of sildenafil-dependent HIF-1α activation. In conclusion, the therapeutic effect of sildenafil on neonatal BPD is likely to be attributed to HIF-1 activation.


This work was supported by a grant from the Korean Ministry of Health and Welfare Research Fund 2010 (A100236).

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PC39
Characterisation of vagal afferent-evoked 5-HT release detected by fast-cyclic voltammetry in the nucleus tractus solitarius (NTS) of the anaesthetised male rat
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Vagal afferent activation of NTS neurons is mediated by 5-HT3/7 receptors (Ramage & Villalon, 2008). Using fast cyclic voltammetry it was shown that extracellular 5-HT in the NTS increases in response to vagal afferent stimulation (Millar et al., 2009). Experiments were carried out to investigate the characteristics of this response. Spraque-Dawley rats (200-275g) were anesthetised with α-chloralose (120 mg kg-1, i.v), neuromuscular blocked (α-bungarotoxin 30 μg, i.v.) and artificially ventilated. Depth of anaesthesia was assessed by the stability of BP and HR following a noxious stimulus and additional anaesthetic was given when necessary. The left vagus nerve was exposed and tied distally to the stimulating site. A carbon fibre microelectrode (tip Ø 7 x 100 μm; Millar & Pelling, 2001) was lowered into the NTS until the evoked action potentials from vagal stimulation (1Hz, 100μA, 1ms) were maximal. Recording sites were confirmed histologically. A modified form of fast differential voltammetry was used for electrochemical detection of 5-HT. Electrochemical signals were quantified by differential recording of the peak oxidation and re-reduction currents. All values (n=3-6) are means ± S.E.M. Comparisons between means were made with Student’s unpaired t-test. P<0.05 was considered to be significant.

At threshold (40-100μA) for electrically-evoked activity over the frequency range of 5 to 50Hz no 5-HT could be detected. At 5x threshold there was a frequency dependent increase in
the 5-HT concentration, ranging from 6±2 to 22±8 nM. At 10 & 30x threshold this relationship was not observed, although 50Hz still caused a maximum increase of 17±4 (10x) and 26±10 nM (30x). Stimulation of cardiopulmonary C fibres with phenylbiguanide (50 μg kg⁻¹, i.a.) caused an increased in 5-HT of 7 ± 1 nM. Topical application of CdCl₂ (10 mM, 30μl) attenuated the electrically evoked (20Hz, 10x threshold) increase in 5-HT (3 ± 0.4 c.f. 7 ± 0.6 nM). Changes in HR and BP associated with vagal stimulation were attenuated. Blockade of the serotonin transporter (SERT) with fluoxetine (1 mg kg⁻¹; i.v.) failed to increase release. However, the OCT3 (uptake₂) and plasma membrane monoamine transporter (PMAT) blocker decyinium-22 (D-22, 1 mg kg⁻¹, i.v., Hayer-Zillgen et al, 2002) caused a significant increase in electrically evoked 5-HT (107 ± 52 %) c.f. DMSO (25 μl) control. This was associated with a significant increase (176 ± 84 %) in the evoked bradycardia. Both vagal stimulation and D-22 caused hypertension, however chlorisondamine (1 mg kg⁻¹, i.v.) had no effect on the increase in 5-HT despite blocking the evoked hypotension and causing a fall in BP.

It is concluded that the 5-HT release in the NTS evoked by vagal afferent stimulation is removed by OCT3/PMAT not by SERT.


PSH is in receipt of BBSRC PhD studentship.

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PC40

Evidence that activation of central 5-HT₇ receptors cause sympathoexcitation in anaesthetized rats

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Central 5-HT-containing pathways are known to play a physiological role in the regulation of the cardiovascular system (Ramage & Villalón, 2008). Of the 14 different receptor subtypes, 5-HT₁A, 5-HT₂ and 5-HT₃ have been implicated in this regulation and more recently 5-HT₇ receptors. The present experiments were carried out to further investigate the role of 5-HT₇ receptors by examining the effects of the non-selective 5-HT₇ agonist 5-CT and the selective antagonists SB-269970 and SB-258719 (Hagen et al. 2000) given intracerebroventricularly (i.c.v.). Male Sprague-Dawley rats (230-270g) were anaesthetized with α-chloralose (100 mg kg⁻¹, i.v.), neuromuscular blocked (α-bungarotoxin 30 μg, i.v.) and artificially ventilated. Recordings were made of arterial blood pressure (BP), heart rate (HR) and renal sympathetic nerve activity (RNA). Depth of anaesthesia was assessed by the stability of BP and HR following a noxious stimulus and additional anaesthetic was given when necessary. Changes were compared with saline (5 μl) by two-way ANOVA and the least significant difference test. P<0.05 was considered to be significant. All values are means ± S.E.M.

Cumulative doses of 5-CT (0.01-1 μg kg⁻¹; n=5) caused significant dose related increases in RNA, BP and HR, although at the highest dose (10 μg kg⁻¹) BP fell (24 ± 3 mmHg). Pretreatment for 20 min (100 μg kg⁻¹, n=4) with either SB-269970 or SB-258719 attenuated the effects of 5-CT. Both antagonists caused initial falls in BP (13 ± 2 & 19 ± 2 mmHg) and increases in RNA (27 ± 4 & 59 ± 11 %) as did 10 μg kg⁻¹ of SB-258719, which also blocked the effects of 5-CT. Cumulative doses of either antagonist (0.1-100 μg kg⁻¹, n=4) caused at the highest two doses (10 & 100 μg kg⁻¹) renal sympathoexcitation reaching 151 ± 30 & 83 ± 21 %. For SB-258719 this was associated with falls in BP, while for SB-269970 this only occurred at 100 μg kg⁻¹. HR was unaffected. Pretreatment with SB-269970 (100 μg kg⁻¹, n=4) attenuated these effects of SB-258719. Interestingly, SB-269970 i.v. (100 μg kg⁻¹, n=4) had little effect on RNA and BP as did microinjections of SB-269970 (10 μg kg⁻¹, 1 μl, n=4) into the dorsal raphe.

The data suggests that the activation of 5-HT₇ receptors accessed by i.c.v causes sympathoexcitation and indicates that at this level of the brain the antagonists are having a partial agonist action. Interestingly, when SB-269970 was given intracerebrally there was no effect (Kellett et al. 2005). The ability of these ligands to cause a fall in blood pressure is difficult to explain. For 5-CT this could be due to a peripheral action but this would not apply to the antagonists. Therefore it is suggested that these falls may involve the central release of a vasodilator peptide. The exact central site/s these effects are being mediated by remain to be determined.

Hagen J.J. et al. (2000). Br J Pharmacol 130, 539-548
Kellett DO et al. (2005). J. Physiol. 563,319-331

This work was supported by BHF grant PG/07/119/24169

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC41

Modelling the role of the A-type potassium current in regulating excitability in sympathetic preganglionic neurones

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The sympathetic preganglionic neurone (SPN) is the last central site for integration of sympathetic efferent traffic. As such, the integrative properties are of paramount importance in determining the nature of its output. Intracellular recording studies in vitro and in vivo have provided information about the integrative characteristics of the SPN, although this information has never been used to model the integrative properties of the SPN. We have speculated that changes in excitability of SPN may underpin the increase in sympathetic traffic seen in models of hypertension [1,2] and specifically that an alteration in the A-type potassium current (IA) in these neurons may contribute to this change in excitability. Therefore we have built a NEURON model [3] of the SPN including the following features: medial and lateral dendrites, an axon with Hodgkin-Huxley dynamics, and a soma with membrane conductances such as calcium-activated potassium, N- and L-type calcium and potassium afterhyperpolarisation. We used this model to investigate the role of transient rectifier IA in regulating the excitability of SPN. We tailored a Borg-Graham model of IA to match the known dynamic characteristics.
of experimentally recorded data in rat SPN. Besides alterations of the activation and inactivation parameters and kinetics this also required that the Borg-Graham model of IA be adjusted to allow independent control of the steady-state functions and dynamic time-constants of this current. In silico experiments showed that this refined IA model gave responses similar to those recorded in vitro (see Figure) [4].

Further simulation showed that IA plays an important role in regulating the excitability of SPN and is active at membrane potentials close to rest. Thus, small alterations in the kinetics or density of IA profoundly influence the excitability of SPN with the following features; a reduction in firing frequency in response to an injected current, an alteration in spike accommodation and an alteration in the amplitude and duration of the afterhyperpolarisation. Along with these findings (which are based on preliminary simulations), we found that the output response of SPN to incoming synaptic traffic is dependent on the characteristics of IA. These modelling data are consistent with IA playing a central role in determining SPN excitability and its response to synaptic input, and indicate that changes in its properties could play an important role in elevating the sympathetic nervous system output in hypertensive rats.

Figure: A) SPN model response to a family of injected hyperpolarising current pulses (Ba) Somatic A-conductance response (Ab) The family of current pulses (Ac) Membrane potential response to current pulses - note the inflection in the decay profile, which is characteristic for A-conductant activation. B) Membrane potential response to a step depolarising pulse injection for two SPN models, each with different values of maximal A-conductance density (Ba) Membrane potential response to the injected current - note the increased time-to-fire for the model with larger density (Bb) Injected depolarising pulse.


This work was funded jointly by BBSRC and EPSRC.

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PC42

A micturition switch in the periaqueductal grey matter in rats

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In humans and many socialised animals voiding can be suppressed, even when the bladder is full, until the individual finds itself in a safe and socially acceptable environment. Failure of this inhibitory control mechanism may underlie some forms of urinary urge incontinence.

Afferent signals from the distended bladder activate a spinomidbrain–spinal pathway that initiates co-ordinated activation of the detrusor and external urethral sphincter (EUS) to induce voiding [1]. Critical synaptic relays are present in the caudalmost part of the ventrolateral periaqueductal grey matter (cvlatPAG) [2]. In humans deep brain stimulation (DBS) is increasingly used to manipulate the activity of brain circuits for therapeutic gain [3]. We have investigated whether DBS in the cvlatPAG can suppress voiding in rats.

Urethane anaesthetised (1.5g Kg\(^{-1}\) ip) male Sprague Dawley rats (n=13, 259-403g) were instrumented to record femoral arterial pressure, bladder pressure, EMG from the EUS and urine output. The trachea was cannulated and a venous catheter inserted to administer fluids. Rectal temperature was maintained at 36.5-37.5°C. A needle connected to a t-piece was inserted into the bladder to measure bladder pressure and infuse saline (6ml h\(^{-1}\), 165mM), which induced cycles of filling and voiding (0.41±0.05min\(^{-1}\) means±S.E.M). Each void was accompanied by a sharp rise in bladder pressure and the development of bursting activity in the EUS. An insulated stainless steel stimulating electrode, tip 60μm, was positioned stereotaxically in the caudal midbrain. The cvlatPAG and underlying ventral tegmental area were mapped using a standard stimulus (0.5ms, 60Hz, 180μA for 20s), which was applied within 2s of the onset of the sharp rise in bladder pressure that indicated an imminent void. Stimulation sites were verified histologically from marks of electrode tracks in fixed brain tissue. At 34/72 sites tested, voiding was attenuated (n=19) or completely suppressed (n=15) during the stimulation period. Urine output was reduced from 7.3±0.6 to 1.4±0.3 drops, the accompanying rise in bladder pressure reduced from 19.6±0.8 to 15.0±2.2 mmHg (P<0.05 Student’s paired T-test) and bursting activity in the EUS failed to develop. Stimulation at effective sites was accompanied by a modest pressor response (15.6±1.3 mmHg) and tachycardia (17.9±2.6 beats min\(^{-1}\)). Effective stimulation sites were localised within the ventral half of the caudal PAG at approximately P8.8 [4] and extended into the underlying tegmental field.

At 9 effective sites we investigated the effect of longer periods of continuous stimulation (1-7 min). We were able to suppress voiding completely for up to 7.3 min, despite the continued infusion of saline into the bladder. Our data show that even when the bladder is full, micturition can be deferred by using DBS to modulate activity in the micturition reflex pathway at the level of the cvlatPAG.

**PC43**

Real time monitoring of neurotransmitter uptake and storage in PC-12 cells: implication for co-storage and co-transmission

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Although co-transmission from many autonomic neurons undoubtedly takes place, it is unclear whether neurotransmitter storage takes place within the same or separate vesicles. The use of fluorescent neurotransmitter transporter substrates allows monitoring of vesicular uptake (Parker et al., 2010), indicating the presence of specific vesicles, and may allow the question of co-storage of purines and catecholamines to be definitively answered.

Cultured PC-12 cells were exposed to a fluorescent catecholamine transporter and vesicular monoamine transporter (VMAT) substrate (Neurotransmitter Transporter Uptake Assay; NTUA) using protocols similar to those described by Parker et al. (2010) or the SLC17A9 substrate quinacrine. SLC17A9 has recently been identified as the vesicular ATP transporter, and has hence been called the vesicular nucleotide uptake transporter (VNUT)(Sawada et al., 2008). Fluorescence accumulation was monitored in real time using fluorescence microscopy. NTUA was excited at 405nm; quinacrine was excited at 456nm.

Quinacrine (1 μM) was found to accumulate in a punctuate manner in PC12 cells, (12.4 ± 3.1 granules per cell; mean ± S.E.M.), as was NTUA (1:100) to a similar extent (9.5 ± 1.8 granules per cell; Student’s t-test vs quinacrine, p>0.05, 24 cells, n=4). Accumulation of quinacrine was found to be sensitive to the SLC17A9 inhibitor Evans blue (1 μM; 1.3 ± 0.3 granules per cell; p<0.05, 24 cells, n=4). NTUA accumulation was insensitive to the noradrenaline transporter inhibitor desipramine (1 μM; 8.4 ± 1.2 granules per cell; p=0.05, 22 cells, n=4), but was sensitive to the dopamine transporter inhibitor GBR 12909 (100 nM; 4.7 ± 0.4 granules per cell; p<0.05, 24 cells n=4).

In this investigation, two fluorescent neurotransmitter transporter substrates are evaluated for their specificity. A similar number of vesicles were detected using NTUA and quinacrine, suggesting co-localization or a similar number of granules for ATP and catecholamines. Co-localization experiments, requiring careful spectral separation or novel experimental approaches, may indicate whether or not ATP can be co-stored in vesicles with catecholamines. Altered neurotransmitter release can take place in healthy ageing, and may be a causative factor in a range of CNS, and PNS pathologies (Burnstock, 2007). Targeted modulation of vesicular co-storage may in future offer an alternative therapeutic approach.


Dr Sidaway is supported by the University of Birmingham. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC44**

Manipulation of the cardiovascular response to postural challenge by pedunculopontine nucleus stimulation in humans

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Introduction: Manipulation of cardiovascular performance by electrical stimulation of the brain has been demonstrated in animals and humans. One such site is the mesencephalic loco-motor region, formed by the rostral part of the pedunculo-pontine nucleus (PPN). Dysautonomia is common in Parkinson’s disease and up to 50% of patients suffer from orthostatic hypotension which can be severely debilitating. The aim of this study was to test whether PPN stimulation could modulate the postural cardiovascular response to head-up tilt.

Methods: Patients with in-dwelling bilateral PPN electrodes underwent 80 degree head-up tilt testing with stimulation On and Off, in random order. Percentage change in systolic blood pressure during the first three minutes of tilting was the primary outcome variable. Secondary cardiovascular variables were recorded to indicate the mechanism of any blood pressure changes.

Results: Fall in systolic blood pressure after tilt was significantly smaller with PPN stimulation than without (mean -8.3% versus -17.2%, t=2.679, df=5, p=0.044 using paired samples t-tests). A similar increase in heart rate of 10% occurred in both conditions, dp/dt, a surrogate marker of cardiac contractility, and pulse pressure, a surrogate marker of peripheral vascular resistance, were maintained better with stimulation (p=0.018 and 0.030, respectively). Baroreceptor reflex sensitivity decreased significantly with stimulation (z=-1.992, p=0.046 using Wilcoxon signed ranks test). Analysis of the electrocardiogram at rest revealed no changes in the On compared to Off states in RR-interval, PR-interval or QT variability (n=10, p=0.697, p=0.952 and p=0.897, respectively using paired samples t-tests).

Conclusions: PPN stimulation can modulate the cardiovascular response to head-up tilt in humans. The PPN appears to be influencing systolic blood pressure by increasing peripheral vascular tone and cardiac contractility in addition to alteration of the baroreceptor reflex itself. The mechanism of blood pressure maintenance by PPN stimulation does not appear to be via detectable chronotropic effects.

A grant for this study was received from Medtronic S.A. Oxford Functional Neurosurgery is supported by the Oxford Biomedical Research Centre of the NIHR.
Using a novel decerebrate arterially perfused preparation of the whole rat, the micturition reflex was evoked by fluid infusion into the bladder and non-micturition contractions (NMCs) were characterised. Female Wistar rats (40-80g) were anaesthetized with 2.4% halothane. The bladder was accessed via a laparotomy. The stomach, spleen and intestine were tied and removed. The animal was immersed in cold artificial CSF and decerebrated, at which point anesthesia was withdrawn. The preparation was perfused with carbogenated aCSF (32°C) via a double lumen cannula inserted into the aorta. The heart resumed beating and rhythmic respiratory muscle contractions began within minutes, as perfusion pressure reached 70mmHg. A suction electrode recorded phrenic nerve activity. A cannula, connected to a 3-way tap was inserted into the bladder for filling and pressure monitoring. A bipolar suction electrode recorded external urethral sphincter (EUS) EMG. A bladder for filling and pressure monitoring. A bipolar suction electrode recorded external urethral sphincter (EUS) EMG. A second bipolar electrode measured pelvic bladder afferent nerve activity. All preparations showed characteristic rat filling and voiding responses lasting for 4 hours. NMCs were observed during basal conditions, with frequency and amplitude of 1.4±0.9/min and 3.6±0.5mmHg, respectively, and a duration of 10.9±0.7s (n=12). NMCs followed waves of peristaltic contractions that extended down the length of the ureters as fluid was passed from the kidneys. During slow rates of bladder distension (infusions <20μl/min), NMC frequency was higher as compared to fast infusion rates (infusions >80μl/min). Systemic application of M1 antagonist, pirenzpine abolished NMCs (n=2), although normal voiding responses remained, indicating the contribution of urothelial muscarinic receptors in NMC generation. Application of muscle relaxants, vencuronium bromide (2μg/ml) on EUS decreased the amplitude of NMCs, resulting in leakage of urine likely due to lack of resistance. NMCs triggered bursts of discharge on the pelvic afferent nerve. Nociceptive c-fibre stimulation (with intravesical capsaicin) caused large amplitude NMCs (14.5±±0.8 mmHg compared with paired control 2.3±±0.4mmHg; P=0.0001; t-test) and associated tonic EUS firing. Subsequently, NMC and EUS activity decreased, as a result of desensitisation. Ganglion blockade by hexamethonium (330μM) increased NMC amplitude (but not frequency) by 86%, suggesting tonic inhibition by higher centers. Moreover, when brainstem control had deteriorated NMC amplitude (but not frequency) also increased (5.8±±0.4 mmHg; P=0.0001). These results confirm that NMCs appear to be locally generated via mediator release from urothelial cells. They may serve as an assessment reflex during bladder distension relaying bladder fullness levels to higher centres. However, NMCs are modulated by the CNS which may prevent them from triggering a void until a volume threshold is reached.

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Poster Communications

Contribution of central and peripheral mechanisms for non-micturition contractions in rat bladder function

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PC45

Functional and molecular defects of baroreceptor neurons in genetic hypertension

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We have reported that important determinants of baroreceptor nerve activity include the mechanosensitive Acid Sensing Ion Channel–ASIC2 (Neuron 2009), the calcium-activated K+ channel-BK (J Physiol 2007) and Na+K+ATPase (Am J Physiol 1984). Our goal was to test the hypothesis that genetic dysregulation of these and other ionic events in baroreceptor neurons account for the impaired baroreceptor nerve activity in the spontaneously hypertensive rat (SHR). We injected the red fluorescent dye (Diil) into the wall of the aortic arch of anesthetized (Ketamine/Xylazine 91/12.5 mgm/kg I.P.) SHR and Wistar Kyoto (WKY) control rats. The arch contains the sensory terminals of aortic baroreceptor neurons which reside in the nodose ganglia. Ten days later the ganglia were surgically removed from the deeply anesthetized rats (1cc isoflurane in 1,000cc air) and their cells were dispersed in culture to identify the labeled baroreceptor neurons. Electrophysiological responses to mechanical stimulation (puffing of saline at 13 psi from a pico-pump) and to depolarizing current injections (1-10nA) were obtained with sharp microelectrodes. SHR neurons had a more negative resting membrane potential (RMP) than those from WKY (-61.3±±2.7, n=17 vs. -43.9±±1.9 mV, n=24*); were not depolarized by mechanical stimulation (Δ±±2.1±±0.0 mV, n=15 vs. Δ±±6.8±±2.4 mV, n=20*), and did not fire action potentials during 1nA current injections (0.5±±0.3, n=16 vs. 5.8±±2.7 spikes/s, n=22*) or during their impalement with the microelectrode (0.5±±0.1, n=18 vs. 175±±63 spikes, n=19*) (“p<0.01). PCR arrays of nodose ganglia showed that only 2 genes (Atp1b1 and Atp1b2) were upregulated in SHR vs. WKY by 3.0 and 9.3 fold respectively. These genes encode Na+K+ATPase alpha and beta polypeptides. Among the downregulated genes in SHR, Accn1 (ASIC2) was reduced by 2.1 fold; knmn1b (BK beta1) by 3.7 fold; and several voltage-gated K+ channels (e.g. Kcnca1 / Kv1.1; Kcnna4 / Kv1.4; Kcnj4 / Kir2.3; Kncd2 / Kv4.1-A current) by -1.9, -2.7, -6.7, -2.5 fold respectively. mRNA (qRT-PCR) and proteins confirmed the directional changes in gene expression seen in PCR arrays in SHR. microRNAs which are putative inhibitors of the genes mentioned accounted for some of the changes in gene expression. Ten miRNAs were upregulated and nine were downregulated more than 4 fold. Of these, the upregulated miR34c correlates with decreased ASIC2 and the downregulated miR-29a, miR-29c, miR-142-3p, miR-181a-1, miR-181c, miR-1236 may explain the overexpression of Na+K+ATPase. The results suggest that the molecular profile of enhanced Na+K+ATPase and reduced ASIC2, BKbeta1 and Kv may explain the more negative RMP, and decreased mechanosensitivity and excitability of SHR neurons. Thus we have identified molecules responsible for defective sensory signaling of baroreceptor neurons in SHR. Therapies targeting these molecules may restore baroreflex sensitivity and reduce blood pressure and mortality in hypertension.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC46

Poster Communications

PC46
BDNF-Induced Ca\(^{2+}\) Oscillations in Rat Organotypic Spinal Cord Slices and their Possible Relevance to Neuropathic Pain

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Peripheral nerve injury promotes the release of brain derived neurotrophic factor (BDNF) from spinal microglia and primary afferent terminals. This induces changes in the properties of neurons in the dorsal horn that lead to the ‘central sensitization’ that underlies neuropathic pain. In accordance with procedures approved by the University of Alberta Animal Care and Use Committee, we used defined medium organotypic cultures of rat spinal cord slices to examine changes in the properties of dorsal horn neurons produced by 6d exposure to 100-200ng/ml BDNF (Lu et al., 2009). This mimics the prolonged increase in dorsal horn BDNF levels that accompanies peripheral nerve injury. All BDNF treated cultures exhibited synchronous oscillations in cytosolic Ca\(^{2+}\) concentration as monitored by fluo-4 confocal Ca\(^{2+}\) imaging. These occurred at 0.14±0.01Hz (n=7) and were synchronous with extracellularly recorded field potentials. Oscillations in BDNF-treated slices were eliminated by removal of extracellular Ca\(^{2+}\), or by superfusion of 200μM Cd\(^{2+}\), 0.1μM TTX or 10μM NBQX. This indicates the involvement of voltage gated Ca\(^{2+}\) and Na\(^{+}\) channels, the release of glutamate and the participation of AMPA receptors in the oscillations. The NMDA receptor blocker AP-5 (50μM) reduced the amplitude (P<0.001, unpaired T-test) but not the frequency of oscillations and the Ca\(^{2+}\) permeable AMPA receptor blocker, IEM1460 (50μM) reduced both amplitude and frequency (P<0.001 for both unpaired T-test). Oscillations were augmented by bicuculline 10μM + strychnine 1μM and eliminated by 1mM GABA. Activation of astrocytes with 10μM phenylephrine (Ruangkittisakul et al., 2009) promoted elevation of Ca\(^{2+}\) in populations of cells distinct from those displaying oscillations. The possible contribution of gap junctions was inferred from an increase in width of each oscillation in the presence of carbeneboline (100μM). These findings suggest that BDNF oscillations are primarily neuronal in origin and involve glutamatergic transmission. We also found that Ca\(^{2+}\) signals produced by acute superfusion of 10μM AMPA or 50μM NMDA were augmented in BDNF treated cultures. This suggests that the BDNF-induced oscillations may result, at least in part, from increased neuronal sensitivity to glutamate. This type of long range oscillatory activity may enable interactions between different sensory modalities within the dorsal horn and may thereby contribute to the etiology of nerve-injury induced neuropathic pain.

Lu VB et al. (2009). J Physiol (Lond) 587, 1013-1032


Supported by Canadian Institutes of Health Research

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A non-enzymatic transport metabolon enhances lactate flux in astrocytes

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High-energy metabolites, such as lactate, pyruvate, and ketone bodies, are transported into and out of cells via monocarboxylate transporters (MCT, SLC16), of which 14 isoforms have been described. In the brain, astrocytes express MCT1 and 4, which have been suggested to be responsible for the export of lactate to provide neurons with the energy substrate during increased activity. We have recently shown, that carbonic anhydrase isofrom 2 (CAII) can increase lactate-induced acid/base flux via MCT1 and 4, heterologously expressed in Xenopus oocytes, in a non-catalytic manner (1, 2, 3). In the present study, we tested whether carbonic anhydrase can facilitate lactate transport in astrocytes. Our experiments revealed, that knock-out of CAII induced a significant reduction in the rate of lactate-induced acidification in mouse cerebellar astrocytes, as measured by in situ live-cell imaging with the pH-sensitive fluorescent dye 2’7’-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF) in acute cerebellar slices of CAII\(^{-/-}\) and CAII\(^{-/-}\) mice (B6.D2-Car2n/J). In contrast to the knock-out, blocking CA catalytic activity with 6-Ethoxy-2-benzothiazol-sulfonamid (EZA, 10 μM) had no effect on H\(^+\) flux, suggesting a non-catalytic facilitation of transport activity by CAII. The data could be confirmed by uptake experiments in cultured astrocytes of NMRI mice: Knock-down of CAII and CAIV by siRNA led to a significant decrease in lactate flux, while inhibition of CA catalytic activity with EZA (30 μM) had no effect. To check for a direct interaction between MCT1 and CAII in astrocytes, we applied an in situ proximity ligation assay, which indicated close proximity (< 40 nm) of MCT1 and CAII as intrinsically expressed in cultured mouse astrocytes. Co-immunoprecipitation with CAI and the C-terminal of MCT1 (MCT1-CT), fused to GST, revealed direct binding between MCT1-CT and CAI. To identify the binding site for CAII, we carried out single site mutations of MCT1, heterologously expressed in Xenopus laevis oocytes. The interaction between MCT1 and CAII seems to be facilitated by a cluster of three glutamate residues in the C-terminal of MCT1. While catalytic activity of CAII is apparently not necessary for enhancement of MCT1 transport activity, removal of the intramolecular H\(^+\)-shuttle, His64, in CAI abolished the interaction between the two proteins in Xenopus oocytes. Our results suggest that CAII, directly bound to MCT1, can facilitate lactate flux in astrocytes by acting as a “proton collecting antenna” for the transporter. By this mechanism CAII would facilitate proton movement at the pore of the transporter, suppressing the build-up of proton microdomains, and thereby increasing trans-membrane lactate flux in astrocytes.

This work was funded by the Deutsche Forschungsgemeinschaft DFG (De 231/24-1) and the “Research Initiative Membrane Biology”.


Changes of sleep structure after different types of cold acclimation in rats

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Sleep plays a significant role in cold adaptation. It should be noted that cold adaptation in nature is formed by periodic contact with cold factor and takes place on the background of circadian changes in physiological state of the organism. Now two main experimental methods of cold adaptation are widely used: long-term (LTA) and short-term acclimation (STA), which influence on sleep structure is studied insufficiently. Thus, the aim of our work was the study of changes in sleep structure of rats after LTA and STA.

Experiments were performed in Wistar rats (7-8 months, 220-250 g body weight) and were approved by the Committee of Bioethics at IPCSC NAS of Ukraine. The animals were individually kept in the cages in sound-attenuated chamber with 12:12 h light:dark cycle, Ta=22-24°C, with water and food ad libitum. STA was carried out by exposing the rats to a temperature of -10°C for 15 min every hour during the day, for a total nine exposures. This procedure was repeated on the second day. LTA was produced by keeping rats at +4°C for 4 weeks with free food and water access. Brain bioelectrical activity 2 days prior and 2 day after cold acclimations was manually stage scored as rapid eye movement (REM) sleep, slow wave sleep (SWS) or wake in 4-s epochs. Statistical data processing was performed using ANOVA.

LTA led to the significant SWS amount increase (from 54.8 ± 2.7 to 72.5 ± 4.6%) and REM sleep decrease (from 7 ± 1.1 to 10.6 ± 0.6%) by reducing the wakefulness amount (from 38.2 ± 3 to 16.9 ± 6%). It should be noted that sleep in rats, like nocturnal animals, is characterized by greater amount in the light period of the day (60-70%). LTA led to equalization of diurnal animals, is characterized by greater amount in the light period of the day (60-70%).

STA (-12°C) led to the REM sleep increase (from 6.2 ± 1.2 to 12 ± 1.5%) in the light period only due to increasing of the duration of its episodes (from 95.6 ± 9.3 to 154 ± 9.2s) against decreasing of wakefulness amount (from 47.4 ± 11.1 to 26.7 ± 3.6%). SWS amount did not change, but there was increase in the duration of its episodes (from 152 ± 15.6 to 308 ± 9.2s) against the background of reduction of its number (from 51.3 ± 13 to 21.5 ± 2) during light period of the day. No changes in daily SWS amount were found after STA (+10°C). There was only the tendency to reduce SWS amount in the first 3h after STA in the first day of cold exposures. And SWS increase after ending of STA in both first and second day of exposures was observed.

Thus, both types of acclimation change sleep structure and cyclicity in different manner and it seems possible that the mechanisms involved are fundamentally different and depend on character of cold influence (rhythmic character) and degree of temperature load (cold exposure duration).

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PC49

Two forms of action potential latency-walking in rat and mouse small diameter sensory neurones in vitro, discriminated by tetrodotoxin

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Action potentials recorded in IB4+ve small (<25 μm apparent diameter) dorsal root ganglion (DRG) neurones are typically of long duration, consistent with a major contribution of the kinetically slow, tetrodotoxin-resistant (TTX-r) transient Na+ current, Na9,1.8, to neurone excitability (e.g. Blair & Bean 2002; Snape et al. 2010). Action potential latency-walking elicited by low frequency stimulation (2 Hz) in these neurones is probably caused by a progressive slow inactivation of Na+ currents, and we wished to confirm whether properties of either Na9,1.8 or TTX-sensitive (TTX-s) Na+ currents (or both) might explain the phenomenon. We first investigated the effects of a small molecule blocker of Na9,1.8 (A-803467) that is reported to show channel sub-type selective block (Jarvis et al. 2007), and we found that A-803467 could not select convincingly between Na+ currents recorded in different small diameter rat sensory neurones in voltage-clamp (fractional block by 300 nM = 0.60 ± 0.09 and 0.49 ± 0.03 (mean ± SEM) for transient TTX-s and TTX-r Na+ currents, recorded in small diameter IB4- and IB4+ve neurones, respectively, n = 3, 3; P = 0.34, t-test), and we did not investigate it further. However, we also found that application of TTX (250 nM) in current-clamp enabled us to discriminate two forms of latency walking in neurones with long duration action potentials. In neurones with stable resting potentials of -60 mV or more negative, one form was typified by a consistently less than 1.5 ms increase in action potential latency in response to 100 stimuli at 2 Hz (n = 11), and this was unchanged by exposure to TTX (n = 4, P = 0.24, paired t-test). However, walking was also recorded that could be considerably greater than 1.5 ms (n = 4), and the latency change in these neurones was substantially reduced by TTX, Figure 1.

We conclude that at least one TTX-s Na+ channel subtype and probably Na9,1.8, contribute to action potential latency walking, with the activity dependent loss of TTX-s channels most dramatically affecting latency. We suggest that varying degrees of activity dependent changes in C-fibre conduction time may reflect the differential expression of Na+ channel subtypes in axons.

Figure 1. Action potential latency walking in response to 2 Hz stimulation in an IB4+ve mouse DRG neurone, in the absence (A), and presence (B) of 250 nM TTX. Responses to stimulus number 10, 20, 40 and 100 are shown.

Neuronal over-expression of Neuregulin-1 slows carbachol-induced gamma oscillations and alters GABAergic inhibition in hippocampus

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The neuronal growth factor Neuregulin 1 (NRG1) and its receptor ErbB4 are involved in neurodevelopment and postnatal synaptic maturation. Behavioural phenotyping of transgenic mice that over-express type I isoform of NRG1 (NRG1tyI) under the Thy1 promoter (1) has revealed an age-emergent impairment of hippocampal-dependent spatial working memory. The underlying neurobiological changes are unclear and we therefore studied the role of increased NRG1tyI expression on hippocampal network functioning in the transgenic mice (1-6 months old). We discovered changes in carbachol-induced hippocampal gamma oscillations (20-80 Hz) and in the rhythmic GABAergic inhibition that entrains CA3 pyramidal cells to the gamma rhythm. Recorings were made from 350-400μm thick acute hippocampal slices, prepared from mice terminally anaesthetised with pentobarbitone sodium (dosage ~0.2mg/g, i.p.). Gamma oscillations were induced by application of 5-20μM carbachol. The peak frequency, but not the power, of oscillations in the CA3 area was significantly reduced in transgenic mice (mean frequency ± SEM: 22.1±0.5 Hz, n=34 slices) compared to wild-type littermates (26.4±0.5 Hz, n=46 slices, Mann-Whitney U Test, p<0.001). Action potential firing of CA3 principal cells was similarly phase-locked to the field potential gamma cycles in both genotypes. However, the dynamics of inhibitory synaptic currents in CA3 pyramidal cells during the gamma oscillation were sustained in the NRG1tyI mice compared to wild type littermates (half amplitude decay time in NRG1tyI: 12.5±0.5 ms, n=8 cells vs wt: 10.3±0.2 ms, n=13 cells, independent sample T test, p<0.001). In contrast, excitatory current kinetics were not altered. These findings suggest that the local inhibitory GABAergic circuits responsible for the rhythmic inhibition are changed in the mice over-expressing NRG1. Because Parvalbumin-positive (PV+) interneurons are crucial for both working memory (2) as well as gamma oscillations (3), and are a likely primary target of NRG1 signalling (4), we selectively study PV+ interneuron-mediated inhibition in the CA3 circuit. We used optical stimulation of light-gated channelrhodopsin-2 (ChR2) selectively expressed by PV+ axons: NRG1tyI mice crossedbreed with PV-Cre mice were exposed to inhalation anaesthesia with isoflurane (induction 4%, maintenance 1.5-2% in O2) and injected with adeno-associated virus encoding flexed ChR2-YFP (yellow fluorescent protein) under stereotaxic guidance. Following in vivo expression of the viral vector for 10-21 days, recordings were made in acute hippocampal slices. Using this approach, we aim to uncover mechanisms underlying altered inhibitory signalling onto pyramidal cells in NRG1tyI transgenic mice.


WN is grateful to the MRC and the Department of Pharmacology for a studentship.

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Behavioural and Neurophysiological Thresholds for Two Pitch Discrimination Tasks


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Pitch perception is an essential part of vocal communication in many species. Appropriate animal models of human pitch perception are needed in order to examine pitch processing at the cellular level. Human pitch thresholds have often been compared to those of other mammals, but not on the same tasks. In animals, pitch thresholds are measured on a Go/No-Go (GNG) task in which the animal must detect a pitch change in a sound sequence. Human pitch thresholds, on the other hand, are measured on 2-Alternative Forced Choice (2AFC) tasks, which usually require subjects to label a pitch shift across 2 sounds as “increasing” or “decreasing”. Here, we compared the pitch thresholds of 5 ferrets and 5 human listeners on a GNG task with thresholds from the same 5 humans and 3 other ferrets on a 2AFC task. Stimuli were artificial vowels, with a 400-Hz reference. We also examined how the responses of ferret auditory cortical neurons to the same sounds may support performance on both these tasks. Cortical recordings were carried out in 5 ferrets under general anaesthesia (0.022 mg/kg/h i.v. medetomidine, with 5 mg/kg/h i.v. ketamine). P-values for Wilcoxon Rank Sum Tests are reported. The discrimination thresholds of ferrets were poorer on the 2AFC task than the GNG task (p = 0.036), while humans performed similarly on the two tasks (p = 0.063, Figure 1). Humans outperformed ferrets on the 2AFC (p = 0.036) task, but performance on the GNG task did not differ across species (p = 0.056). We designed neurometric algorithms that used the recorded responses of auditory cortical neurons to either (a) detect a change in the pitch of two artificial vowels, or (b) determine the direction of a pitch shift across two vowels. We found that among the neural populations (n = 47) which could perform the pitch discrimination tasks to criterion, pitch thresholds did not differ across the two tasks (p = 0.115, Figure 2).

Human listeners, as well as ferret auditory cortical neurons, provided similar thresholds across the two pitch discrimination tasks. These results suggest that the large difference in ferrets’ performance on the GNG and 2AFC task is mainly due to differences in the tasks’ cognitive demands, rather than their perceptual demands. This study emphasizes the importance of task design in sensory neuroscience, in both estimating perceptual thresholds and in interpreting neurophysiological responses.

Poster Communications

87P
Mental retardation (MR) affects 2-3% of the population, with X-linked mutations a common cause of moderate to severe MR (Ramakers, 2002). OPHN-1 (Ophn-1 in mice) is one of the genes implicated in X-linked mental retardation (XLMR), encoding oligophrenin-1, a RhoGAP protein. Loss of function mutations affect Rho GTPase-dependent signalling pathways and alter actin cytoskeleton dynamics which affect vesicle dynamics and dendritic spine structure, the site of neurotransmission (Khelfaoui et al., 2007). Recent research has suggested oligophrenin-1 regulates endocytosis of synaptic vesicles (Khelfaoui et al., 2009). At present, no drug treatment is available for MR and treatment is primarily through educational therapy. The mechanisms underlying the cognitive decline in MR are poorly understood and better knowledge may enable better pharmacological intervention. Ophn-1 mice (male,3-10 weeks old) were anaesthetised by intraperitoneal injection of medetomidine (1mg/kg) and ketamine (76mg/kg). Values are expressed as mean±S.E.M, analysed by ANOVA, Mann-Whitney U or Student’s t-test. To investigate the role of oligophrenin-1 in synaptic function, extracellular postsynaptic field potentials (PSPs) were recorded from the stratum radiatum in the CA3c region of the hippocampus and evoked by stimulating the hilus. The amplitude of the PSP increased with larger stimulus intensities; Ophn-1+/y responses were smaller than Ophn-1−/y slices (p=0.011, n=5&12, respectively). To investigate the role of oligophrenin-1 in secretory vesicle availability at frequencies relevant to cognition, synaptic responses to repetitive stimuli (40 stimuli at 33Hz) were examined. Ophn-1+/y slices showed less potentiation than Ophn-1−/y slices (p=0.001, n=5&12, respectively). To further elucidate the synaptic changes associated with oligophrenin-1 loss of function, whole cell patch-clamp recordings were used to examine synaptic activity of CA3c pyramidal neurons. The frequency of spontaneous EPSCs and IPSCs (excitatory postsynaptic potentials and inhibitory postsynaptic potentials) was lower in Ophn-1−/y than Ophn-1+/y neurons (sEPSCs: 1.67±0.37 Hz, n=9; 6.91±1.50 Hz n=8, p=0.003; sIPSCs: 8.78±0.81 Hz, n=7; 12.63±0.76 n=7, p=0.009, respectively). The ability of synapses to follow high frequency stimulation (33Hz) was examined. IPSCs built up with successive stimuli, reaching a steady level within 10 stimuli in Ophn-1−/y neurons; IPSC facilitation was much weaker in Ophn-1−/y than Ophn-1+/y neurons. (p=0.001, n=9&17, respectively).

This study demonstrates a synaptic malfunction which may be due to altered vesicle dynamics at the presynaptic terminal as a result of reduced readily releasable pool size or impaired endocytosis, although alterations in AMPA receptor dynamics or long term depression may also contribute (Khelfaoui et al., 2009).


Supported by a Wellcome Trust grant to JGR (074771/Z/04/Z)
into the brain because of the blood brain barrier. Unfortunately, glioma can regenerate even after the treatment.

Liposome has the interesting properties of entrapping hydrophilic and hydrophobic drugs and targeting tumours cells. Liposomes are vesicles made of phospholipid molecules which are similar to biological membranes and hence bio-compatible and biodegradable (Katari et al., 1990; Crommelin and Sindelar, 2002). The major drawback of conventionally prepared liposomes is that, they are chemically and physically unstable and they are difficult to manufacture on a large scale. Stability problems can be avoided by formulation of liposomes using the solvent based proliposome method (Perrett et al., 1991). The resultant liposomes provide high entrapment of hydrophilic agents and can also be prepared on a large scale. The aim of our project is to use herbal extracts such as Taxol, Crude M. Charantia and Alpha-beta momorcharin binded to the phosphatidylcholone to manufacture solvent-based proliposome which can be used to generate liposomes when the aqueous phase is added. The resultant size of the vesicles was compared with the conventional method of producing liposome and also with the increasing concentration of the model anticancer drug. The efficacy of the anticancer-liposome formulations were investigated for the viability of normal glial cells (SVGP12) and glioma cell lines (1321N1, G5o-3 and U87-MG) using the proliposome method. On measurement of ATP release by SVGP12, 1321N1, G5o-3 and U87-MG after treatment with anticancer liposomes showed more significant growth inhibition of glioma cell line without effecting the growth of the glial cells. Further experiments are required to determine the mechanism action of anticancer liposome in inhibiting glioma.


Garside R, Pitt M, Anderson R (2005). Glioma (newly diagnosed and high grade) - carmustine implants and temozolomide: appraisal con-


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PC55

Role of Ryanodine Receptors, IP3 Receptors, and Phopholipase C in Facilitation and Tetanic Depression at the Frog Neuromuscular Junction

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At the frog neuromuscular junction, calcium-induced calcium release facilitates EPP amplitudes in conditions of low Ca2+ and high-Mg2+ (Narita et al, 2000), whereas there have been no reports of a role for IP3 receptors in short term plasticity at this synapse. Here we investigated the role of these receptors in short-term plasticity recorded in Ringer containing a physiological concentration of calcium ion. Experiments were performed on isolated nerve muscle preparations from young frogs of either sex weighing 70-90 g. The animals were sacrificed by double pithing according to local animal care guidelines. Intracellular recordings of end-plate potentials (EPPs) in response to suprathreshold nerve stimulation were obtained from cutaneous pectoris or sartorius muscle fibers in normal amphibian Ringer containing d-tubocurarine (6 μM) to block muscle contractions. Muscles were treated with either ryanodine (10 μM), 2-APB (10 μM) or U-73122 (5 μM) to block ryanodine receptors and calcium-induced calcium release, IP3 receptors or phospholipase C, respectively. Control recordings were obtained in the corresponding drug vehicles (0,1% ethanol, 0,002% methanol, 0,05% DMSO, respectively). Treatment with ryanodine caused a decrease in paired-pulse facilitation for intervals < 20 ms and a marked decrease in the extent of tetanic depression. Treatment with 2-APB caused a reduction in EPP amplitude, increased paired-pulse facilitation for intervals < 20 ms, and significantly reduced the extent of tetanic depression. Treatment with U-73122 caused a decrease in EPP amplitude, decreased paired pulse facilitation for intervals < 20 ms, and increased the extent of tetanic depression. We conclude that ryanodine receptors, IP3 receptors, and phospholipase C contribute to short-term plasticity at the frog neuromuscular junction.


Supported by CNPq and Fapemig, Brazil

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PC56

Multi Electrode Array recordings from the striatum and adjacent anatomical areas

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Multi Electrode Arrays (MEAs) provide a way of recording extra-cellular action potentials from many tens of sites within a sample, but their use with acute slice preparations has been limited by low signal-to-noise ratios as a result of poor adherence between the slice and substrate, and by the degradation of samples due to the poor access of superfusate to the underside of the sample. Perforated MEAs solve both of these problems by allowing negative pressure to be applied perpendicular to the recording plane of the array, providing excellent proximity between slice and electrodes and maximising the access of superfusate to the sample. Presented here is a cheap and reliable gravity-fed negative pressure double superfusion system for use with Multichannel Systems[1] perforated MEAs and baseplates that easily and reliably records spikes with magnitudes of at least 4 standard deviations above noise and for durations of up to 24 hours. Using this system, spike trains were recorded from the cortex, striatum, and the globus pallidus of parasagittal slices taken from the brains of p14-p30 Sprague Dawley rats. Recordings of tonically active neurons within each region were easily obtained on multiple channels, making this technique a higher-throughput alter-
native to traditional methods of in vitro extracellular recording such as low impedance glass electrodes or cell-attached recording.

http://www.multichannelsystems.com/

PhD funded by the BBSRC. Equipment generously loaned by Scientifica, Ltd. Pilot work performed by Roosa Tikkanen.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC57**

Brain microcirculation is regulated by neuronal-derived nitric oxide: real time and in vivo demonstration

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The neurovascular coupling (NVC) process establishes a functional bridge between active neurons and local blood flow, hence providing working neurons with energy substrates, glucose and oxygen. Although it is accepted that glutamate stimulus is a required trigger, the mechanistic details of such a process have remained controversial, largely because of experimental difficulties in addressing the problem in a real time, quantitative and dynamic fashion in vivo.

Previous published evidences suggest that increased CBF occurs following glutamate NMDA receptor activation and that nitric oxide (NO) is a likely candidate to mediate NVC. Nitric oxide is produced upon glutamate-dependent neuronal nitric oxide synthase (nNOS) activation and is able to diffuse, generating a volume signaling that affects a large population of neighboring cells. Using a tri-component microsensor array with a versatile geometry and consisting of NO-selective micro-electrode, an ejection pipette and a laser Doppler sensor inserted stereotaxically in the brain of anesthetized Wistar rat (via an intraperitoneal injection of urethane 1.25 g/kg) we show that a transient increase in NO production induced by an ejection 0.5 nmol glutamate (25 nL) during 1 s in a defined area of hippocampus, is followed, seconds later, by a transient change in cerebral blood flow (CBF). NO may peak at about 1 µM with a time rise of 22 ± 3 s and a total duration of 64 ± 4 s (15 individual experiments). The CBF increased 7 ± 2 s after stimulation reaching 122 ± 5% of the basal level after 62 ± 3 s and returning to basal levels after 216 ± 15 s. Blocking the NMDA receptor (MK-801 (1 mg/kg) elicited both an inhibition of NO production (73 ± 8%, \(p = 0.0017\), \(n = 3\)) and a decrease of glutamate-induced CBF (74 ± 3%, \(p = 0.0008\), \(n = 3\)). Similarly, the inhibition of the neuronal isoform of NOS by 7-NI (50 mg/kg) induced an inhibition of both, NO and CBF responses to glutamate (62 ± 10 and 83 ± 6%, \(p = 0.0002\) and \(p = 0.0025\), respectively, \(n = 4\)). A local and transitory elevation of O2 tension was observed following the increase in CBF. In summary, we have shown in vivo in rat brain and in a real-time and dynamic fashion that: 1) upon stimulation with glutamate, a transient increase in NO concentration is observed, 2) the dynamics of NO signal precedes that of CBF change which, in turn is followed by a transient increase in O2 tension, 3) both, NO, blood flow changes, may be pharmacologically modulated and are coupled in terms of time, space and amplitude. Thus, neurovascular coupling is mediated by nNOS-derived NO via a diffusional wireless connection between active glutamatergic neurons and blood vessels.

This work was partially supported by projects SAU-BEB/103228/2008 and PTDC/SAU-NEU/108992/2008 from FCT (Portugal).

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**PC58**

Extensive cortical cholinergic innervation is required for normal auditory processing and perceptual learning in adult ferrets

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Cholinergic modulation of cortical activity has been implicated in a number of cognitive functions, including the induction of experience-dependent plasticity, processing of sensory inputs and the potential mechanisms underlying attention. Given the clear role for acetylcholine in altering cortical activity, and the knowledge that an intact auditory cortex is critical for many aspects of auditory processing, including the perception of sound source location, we investigated the role cortical acetylcholine efflux plays in modulating auditory processing in adult ferrets.

Anatomical studies revealed the distribution of cholinergic cells in the nucleus basalis (NB), the main source of cholinergic innervation to the cortex, while tracing studies confirmed that neurons within this region project unilaterally to the auditory cortex. Cortical cholinergic input was subsequently disrupted by selectively destroying cholinergic projections from the NB, prior to testing animals on a free-field sound localization task. Ferrets were anaesthetized with ketamine (5 mg/kg i.m.) and medetomidine (0.022 mg/kg i.m.) prior to bilateral injections of either the cholinergic neurotoxin ME20.4-SAP (n=6), or ACSF (n=2), in the NB. ME20.4-SAP injections produced a decrease in the number of cholinergic cells in the nucleus basalis (Controls, 1144±253; Lesion, 550±415), and in acetylcholinesterase positive fibre density throughout the auditory cortex (Controls, density (fibres/mm³) = 0.738±0.069; Lesion, 0.622±0.162).

Animals with cholinergic lesions of the NB were significantly impaired in their ability to localize short duration sounds in azimuth (linear mixed-effects (lme) model, 40-500 ms; \(F(18)=19.57\), \(p<0.01\)). Performance impairments manifested as a decrease in correct responses, and an increase in mean error magnitude, over all speaker locations, and an increase in front-back errors, or mislocalizations to the incorrect antero-posterior hemifield. The degree of performance impairment positively correlated with the extent of cholinergic cell loss in NB (linear model, 40-500 ms; \(F(1.12)=172.72\), \(p<0.001\), with more extensive cell loss producing greater performance impairments. Furthermore, the ability to adapt to perturbations in spatial cue composition induced by unilateral earplugging was impaired. Recovery of function was less complete (unpaired
Nitric oxide production and platelet function in obese adults

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Introduction: Obesity is associated with an elevated risk of cardiovascular morbidity and mortality due to atherothrombotic events (1). Nitric oxide is produced through the conversion of the semi-essential cationic amino acid L-arginine into L-citrulline and NO in a reaction catalyzed by a family of NO synthases (NOS)(2). The biological effects of NO are mainly mediated through cyclic guanosine monophosphate (cGMP), which regulates vascular smooth muscle relaxation, inhibits platelet aggregation and adhesion and induces platelet disaggregation (2). L-arginine is also a substrate for arginase, which competes with NOS for L-arginine (3). Methods: Eleven patients classified as obese according to the National Health Institute – NHI (body mass index > 30 kg/m²) (4), recruited from the Pedro Ernesto Hospital, and eleven age-matched healthy controls were included in the study. The Pedro Ernesto Hospital Ethical Committee approved this study (2488 – CEP/HUPE), and informed consent was obtained from each participant. L-[3H]-arginine influx (1–50 μM) was measured over 5 min and L-leucine (10 mM) was used to resolve total L-arginine transport in platelets into system y+L and transport with diffusion kinetics. Basal NOS activity was determined by the conversion of L-[3H]-arginine to L-[3H]-citrulline. cGMP content was determined in washed platelets at baseline using a commercial ELISA method. Platelet aggregation was evaluated in platelet rich plasma (PRP) by optical densitometry. L-arginine concentration in plasma was measured through the high-performance liquid chromatography (HPLC) method. Arginase activity was determined by the conversion of L-[14C]-arginine to [14C]-urea. Statistical significance was assessed by the Student t-Test, with p < 0.05.

Results: Total L-[3H]-arginine transport (35.57 ± 4.04 vs 61.88 ± 7.47 pmol/109 cells/min, n=11) and transport by y+L system (17.36 ± 2.36 vs 33.37 ± 6.48 pmol/109 cells/min, n=11), NOS activity (0.065 ± 0.009 vs 0.101 ± 0.012 pmol/108 cells/min, n=8) and basal cGMP levels (0.31 ± 0.02 vs 0.50 ± 0.07 pmol/108 cells, n=9) were reduced in platelets. In this context, there was increased platelet aggregation (89.63 ± 8.73 X 76.67 ± 14.27%, n=9) in obese adults compared to control. On the other hand, plasma levels of L-arginine (67.0 ± 9.3 X 70.1 ± 14.7 μM/L, n=7) and arginase activity (0.061 ± 0.007 X 0.08 ± 0.01 pmol urea/mg protein/2h, n=10) were unaltered.

Conclusion: The dysfunction of L-arginine-NO-cGMP signalling observed in platelets of obese patients may contribute to cardiovascular events in these patients.


This work was supported by FAPERJ and CNPq grants.

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Stability of Jurkat cell size in ATP-depleting and furosemide-containing media

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The Na⁺-K⁺ pump is considered to be essential for the control of cell volume by the well-accepted ‘pump-leak’ mechanism by which Cl⁻ influx and osmotic swelling are prevented by membrane polarization. Accordingly, inhibition of the Na⁺-K⁺ pump would lead to cell swelling and lysis. Controlled cell death by apoptosis is accompanied by a decrease in Na⁺-K⁺ pump activity in at least some cell types, including Jurkat cells, but cells typically maintain their volume or shrink rather than swell during apoptosis. Ouabain is a Na⁺-K⁺ pump inhibitor that induces apoptosis without cell swelling in Jurkat cells, suggesting the possibility of an alternative pumping mechanism for extruding osmolytes. Depletion of ATP would be expected to cause cell swelling if volume control was dependent on an ATP-driven pump. Jurkat cells were incubated in control RPMI medium or in ATP-depleting conditions: 1 μM ouabain; glucose replaced with 2-deoxyglucose (2-DG; 10 mM) or 100 μM furosemide (initial control 139 ± 1.1, 117 ± 1.0, 672; 8.5 hr control 123 ± 1.1, 132 ± 1.6, 769 (2-DG + rotenone) and were hardly changed after 5 hr incubation: 125 ± 1.1, 1312 (control) and 119 ± 0.9, 1569 (2-DG + rotenone). By 9 hr in 2-DG + rotenone the cells had condensed nuclei caracterizing cell phenotypes. Digital micrographs were taken periodically and analysed for cell profile area using CellProfiler software. Profile areas (mean ± sem in μm², n) were initially 123 ± 1.0, 1328 (control) and 122 ± 1.6, 769 (2-DG + rotenone) and were hardly changed after 5 hr incubation: 125 ± 1.1, 1312 (control) and 119 ± 0.9, 1569 (2-DG + rotenone). By 9 hr in 2-DG + rotenone the cells had condensed nuclei caracteristic of apoptosis. One alternative mechanism for volume stability in the face of Na⁺-K⁺ pump inhibition depends on concurrent Cl⁻ efflux down an outward gradient set up by Na⁺-Cl⁻ cotransport via carriers such as NKCC. Inhibition of such cotransport would cause cell shrinkage. The profile area of Jurkat cells hardly changed during 8.5 hr incubation with 100 μM furosemide, a NKCC inhibitor (initial control 117 ± 1.9, 502; initial furosemide 127 ± 2.9, 627; 8.5 hr control 123 ± 1.1, 1672; 8.5 hr furosemide 121 ± 1.1, 1454). Cells swelled in hypotonic (122 mosm/kg) RPMI (200 ± 1.4, 1872) and returned to control size within 2.6 hr (134 ± 0.9, 2378). This regulatory volume decrease was prevented by 100 μM ouabain (206 ± 2.0, 1503 at 2.6 hr) despite a lack of effect of 100 μM ouabain on cell size in normotonic RPMI (initial control 139 ± 1.1, 1309; 2.6 hr 139 ± 0.8, 2341). These results provide no evidence for ATP-dependent pumps or furosemide-sensitive Cl⁻ uptake in normotonic volume control in Jurkat cells. Experiments reported here were repeated with similar results.


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Poster Communications

PC61

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PC62

Melatonin induces calcium release from agonist-sensitive cytosolic stores and reduces pancreatic tumour cell viability by altering mitochondrial physiology

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Melatonin is considered a promising antioxidant agent, promoting apoptosis in tumour cells while preserving viability of normal cells. Herein, we examined the effects of melatonin on the pancreatic AR42J tumour cell line in culture. AR42J cells (ECACC N° 93100618) were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FBS and antibiotics (0.1 mg/mL streptomycin, 100 IU penicillin) at 37°C in a humidified incubator (5% CO2). We have analysed cytosolic free Ca2⁺ concentration ([Ca2⁺]c) by single cell imaging analysis, and mitochondrial free Ca2⁺ concentration ([Ca2⁺]m) mitochondrial membrane potential (Ψm) and mitochondrial FAD oxidative state, by confocal microscopy. Loading of cells with the fluorescent ratiometric Ca2⁺ indicator fura-2, with rhod-2/AM, or TMRM was employed to monitor [Ca2⁺]c, [Ca2⁺]m and Ψm, respectively. For FAD determination no dye loading was necessary, and autofluorescence of cells was monitored. We have also analyzed cellular viability, by monitoring of reduction of alamarBlue® employing an ELISA spectrophotometer, and caspase-3 activity, which was calculated from the cleavage of the caspase-3-specific fluorogenic substrate (AGDEVD-AMC) and measured with a fluorescence spectrophotometer.

Our results show that melatonin (at the concentrations 1 μM, 10 μM, 100 μM and 1 mM) induced changes in [Ca2⁺]c, that consisted of single or short lasting spikes in form of oscillations, or a slow transient increase followed by a slow decrease towards a value close to the resting level. Depletion of intracellular Ca2⁺ stores by stimulation of cells with 1 nM CCK-8 or thapsigargin blocked changes in [Ca2⁺]c evoked by melatonin in the majority of cells. Conversely, prior stimulation of cells with 1 mM melatonin, in the absence of extracellular Ca2⁺, inhibited Ca2⁺ mobilization in response to a secondary application of CCK-8 or thapsigargin. Additionally, melatonin induced an increase in [Ca2⁺]m, indicating accumulation of released Ca2⁺ into the organelle. Melatonin also induced depolarization of Ψm and led to a reduction in the level of oxidised FAD. In addition, melatonin reduced AR42J cell viability. Finally, we found a Ca2⁺-dependent caspase-3 activation in response to melatonin.

Collectively, these data support the likelihood that melatonin reduces viability of tumour AR42J cells via its action on mitochondrial activity and caspase-3 activation, by Ca2⁺-dependent mechanisms.

This work was supported by Junta de Extremadura-FEDER (PRI08A018 and GR10010).

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The Prostaglandin E2 type 4 receptor mediates the response to oxidant stress in human airway epithelial cells

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Oxidative stress is implicated in the pathogenesis of many inflammatory pulmonary diseases, including cystic fibrosis (CF). CF arises from mutations in the CFTR anion channel, which can be directly activated by oxidant stress. Delineating how oxidative stress stimulates CFTR in airway epithelial cells is useful, both to increase understanding of airways host defence, and also to suggest therapeutic approaches to reduce the oxidant stress burden in the CF lung. Using the airway epithelial cell line Calu-3, we investigated the hypothesis that H2O2, which stimulates anion efflux through CFTR, does so via production of prostaglandin (PG)E2. Using iodide efflux as a biochemical marker of CFTR activity, we found that the H2O2-stimulated iodide efflux was abolished by the cyclooxygenase (COX) inhibitor indomethacin. The response was also abolished by the selective COX-1 inhibitor SC560, while the COX-2 inhibitor Cay10404 had no effect. Since PGE2 is also produced via the activity of microsomal prostaglandin E synthase-1 (mPGES-1), we also investigated the effects of inhibiting this enzyme. Both Cay10589, an mPGES-1 inhibitor, and Cay10526, which decreases mPGES-1 expression, inhibited the H2O2-mediated anion efflux. Therefore, we conclude that the H2O2-stimulated efflux involves PGE2 production via both COX-1 and mPGES-1 activity, while COX-2 is not involved.

We next wished to investigate how much of the H2O2-stimulated efflux was mediated via the PGE2 subtype 4 receptor (EP4), since this is the only PGE2 receptor subtype capable of mediating a CFTR-mediated anion efflux in these cells. Here we utilised AH23848, a selective EP4 receptor antagonist. In the presence of AH23848, the H2O2-stimulated response was significantly inhibited, effectively to basal levels. In order to investigate EP4 receptor inhibition in a more physiological, polarized system, we also measured the increase in short circuit current (Isc) induced by application of 1 mM H2O2 to the apical face of Calu-3 cells in the presence and absence of AH23848. In these experiments, H2O2 induced a robust increase in Isc, which was significantly attenuated in the presence of AH23848 (22.6 ± 3.0 μA/cm² n = 9 vs 65.3 ± 10.9 μA/cm² for H2O2 alone, n = 12). These results implicate a significant role for the EP4 receptor in mediated the response to anion secretory H2O2, although they do suggest that the complete abolition of the response seen in the non-polarized iodide efflux system may over-estimate its role in the response to oxidant stress. In conclusion, using H2O2 as a model oxidant stress, stimulation of CFTR appears critically dependent both on PGE2 production (via COX-1 and mPGES-1) and the EP4 receptor in Calu-3 airway epithelial cells, a mechanism which would be compromised in the CF airways.

This work was supported by the Canadian Cystic Fibrosis Foundation.

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High-affinity lactate uptake is facilitated by an extracellular, but not by an intracellular carbonic anhydrase

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Lactate transport into and out of cells plays an important role in the distribution of energy-rich compounds within cells and tissues and is mediated mainly by different isoforms of monocarboxylate transporters (MCT). MCT isoforms 1–4 transport monocarboxylates such as lactate together with H+ in an electroneutral transport mode of 1:1 with different substrate affinities. We could recently show that carbonic anhydrase (CA) isoform II, comprised in a family of ubiquitous enzymes catalysing the equilibration of CO2, H+ and HCO3−, enhances transport activity of MCT1 and 4 when expressed in Xenopus oocytes (1, 2). The interaction between MCT1/4 and CAII did not depend on CA catalytic activity, but required the enzyme’s intramolecular H+ shuttle with residue His64 playing a central role (3, 4), suggesting that CAII might act as a “proton collecting antenna” for the carrier to ensure adequate rates of proton-lactate transport.

We have now investigated possible interactions of the high-affinity MCT isoform 2 with cytosolic CAII as well as with extracellular CAIV, anchored to the membrane by a glycosylphosphatidylinositol (GPI) linkage. Activity of the proteins, heterologously expressed or injected in Xenopus laevis oocytes, was determined by measuring changes in intracellular H+ concentration with ion-selective microelectrodes in two-electrode voltage-clamp during application of lactate and CO2/HCO3−. Additionally, expression of MCT2 together with wild-type (WT) or mutants of CAIV was visualised by antibody staining, and possible changes in the level of protein expression were tested by Western blot analyses. Furthermore, catalytic activity of heterologously expressed CAs was determined by mass spectrometry. We could confirm former studies (5) that MCT2 needs embigin as ancillary protein for proper expression and membrane bound CAIV, which would be compromised in the CF airways.

The data suggest that extracellular, membrane bound CAIV augments transport activity of MCT2 in a non-catalytic manner, possibly by facilitating an alternative proton pathway.

Supported by the DFG (GRK 845/3) and the Research Initiative Membrane Transport.


Haplotypedeletion of the gene encoding 11-beta-hydroxysteroid dehydrogenase type 2 (11-β-HSD2+/−) alters the activity of renal serum/glucocorticoid-inducible kinase 1 (SGK1) in mice fed a high Na+ diet

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Na+ re-absorption within the aldosterone-sensitive distal nephron (ASDN) determines the amount of Na+ lost in urine and is therefore critical to whole body Na+ and water balance, and crucial to the control of blood pressure (Loffing & Korbmacher, 2009). This process depends upon epithelial Na+ channels (ENaC) which provide a tightly regulated route for apical Na+ entry in absorptive epithelia. Mice with a haplotype deletion of 11-β-HSD2 display inappropriately high levels of ENAC-dependent Na+ absorption when fed a high Na+ diet and this excessive retention of Na+ leads to the development of severe hypertension. Since the physiological role of 11-β-HSD2 is to “protect” the ASDN from circulating cortisol, this abnormally high level of ENAC activity appears to be an inappropriate response to this hormone (Bailey et al., 2010). Such steroid hormones control ENAC via a mechanism that is dependent, at least in part, upon SGK1, and so we have now assayed the activity of this regulatory kinase in renal tissue from 11-β-HSD2+/− mice and from wild type litter mates that had been maintained on a high Na+ diet for 21 days. Kidneys that had been removed from the mice used in an earlier, physiological study (Bailey et al., 2010) and stored at -80°C, were therefore homogenized in the presence of protease inhibitors and aliquots (80 μg) of extracted protein fractionated by SDS-polyacrylamide gel electrophoresis and subject to Western analysis using antibodies against the total and Thr346/356/366-phosphorylated forms of the protein encoded by the N-myc downstreame reguylated gene 1 (NDRG1), and β-actin. These analyses clearly showed (Fig. 1) that Thr346/356/366-phosphorylated NDRG1 was less abundant in the 11-β-HSD2+/− mice (Fig. 1) and this effect cannot be attributed to differences in the amount of protein loaded onto the gels since the overall abundance of NDRG1, and the abundance of β-actin were very similar (Fig. 1). Since NDRG1-Thr346/356/366 is a physiological substrate for SGK1 but not for other, closely related kinases (Murray et al., 2004), the excessive ENAc activity seen in the kidneys of 11-β-HSD2+/− mice (Bailey et al., 2010) now appear to be associated abnormally low levels of SGK1 activity. Whilst this finding was not anticipated, it is interesting that airway epithelial cells chronically (6 – 7 days) exposed to glucocorticoids also display persistent ENAc activity that is accompanied by a loss of SGK1 activity (Inglis et al., 2009). Glucocorticoid-induced SGK1 activation cannot, therefore account for the sustained activation of ENAc seen in 11-β-HSD2+/− mice.

Western blots showing the abundance of NDRG1-P-Thr346/356/366, total NDRG1 and β-actin in kidneys of wild type and 11-β-HSD2+/− mice. Each lane shows analyses of protein from one individual animal.


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Cannabinoids regulate muscarinic receptor-mediated salivation in submandibular salivary gland

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The ability of cannabinoids to modulate the activity of other receptor types was shown by many research groups (1). During the tasting and chewing of food under the physiological conditions muscarinic (M) receptors are core to the saliva secretion (2). Besides, CB1Rs are known to inhibit ACh release and modulate an autonomic neurotransmission to the submandibular gland (SMG) (3). Thus, in the present study, we aimed to explore in vivo the effects of CB1/CB2, cannabinoid receptors (CBRs) agonist – WIN 55212-2 (WIN, 5-M) on the M receptor mediated-stimulation of saliva outflow and content provided by rat SMG. We also measured in vitro Ca2+-ATPase activities in the SMG acinar cell’s microsomal fraction upon activation CBRs. The male Wistar rats were anesthetized with i.p. injection of pentobarbital (30–40 mg/kg). Saliva was collected using variable speed peristaltic pump. The salivation was evaluated by saliva flow rate and total proteins concentration in saliva collected from the ducts in oral cavity before and after administration of agonists. SMG cells were isolated by collagenase digestion. Stimulation of salivation with i.p. injection of 0.2 and 2 mg/kg M-receptor agonist pilocarpine and simultaneous intraglandular injection of WIN caused the decrease of the stimulation potency of pilocarpine on the rate of salivation by 40±8% (p<0.01, n=6) and 27±6% (p<0.01, n=5) correspondingly. Even more pronounced inhibitory effect of CBRs activation was shown when both agonists were injected intraglandularly: the WIN-induced suppression of pilocarpine-stimulated effect on salivation was 52±10% (p<0.05, n=6) and 45±4% (p<0.05, n=5) respectively. To evaluate an effect of CBRs
activation on ACh-induced salivation, we first applied to the SMG WIN (for 10 min) and subsequently ACh (5uM). Prolonged application of WIN leads to suppression of basal salivation occurring at 5 and 10 min by 38±7% (p<0.05, n=6) and 47±2% (p<0.05, n=5) vs. the saline-treated group respectively. Pretreatment with WIN significantly inhibited the ACh-induced salivation at 5, 10 and 15 min after ACh administration: the saliva flow rate decreased by 48±7% (p<0.05, n=6), 54±13% (p<0.05, n=6) and 49±5% (p<0.05, n=4) vs. the control group respectively. The latter was accompanied by significant increase of the total protein concentration in the secreted saliva at 5, 10 and 15 min by 37±3% (p<0.05, n=5), 58±5% (p<0.05, n=5) and 56±6% (p<0.05, n=5) vs. the control group respectively. Besides, we also showed that CBRs-induced inhibition of the resting salivation is accompanied by the decreased activity of Ca2+-ATPases in endoplasmic reticulum (by 44±6%, p<0.05, n=6) and increased - in plasma membrane (by 26±6%, p<0.05, n=6). These findings revealed the novel function of CBRs as the negative regulators of agonist-induced stimulated salivation that occurs with the changes of the Ca2+ homeostasis in SMG cells.


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PC67

Novel mutations of CLCNKB gene in patients with Bartter syndrome: a functional analysis in Xenopus laevis oocytes

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Bartter’s syndrome (BS) is an autosomal recessive, salt-wasting tubulopathy characterized by hypokalaemic metabolic alkalosis and secondary hyperaldosteronism (1). BS results from loss-of-function mutations in genes encoding the transport proteins NKCC2, ROMK, CIC-Kb or Barttin (2), which all are involved in NaCl reabsorption in the thick ascending limb of Henle’s loop (TAL). To date, mutations in the CLCNKB have been much less investigated than other BS gene mutations. Here, we investigated 6 novel (L81P, G424R, R351P, A204T, L439P) and 2 published (A204T, R438H) (3, 4) CLCNKB mutations.

The cRNAs for Barttin and FLAG-tagged, wild-type and mutant CIC-Kbbs were injected in Xenopus laevis oocytes. CIC-Kb currents were measured using two-electrode voltage-clamp, and surface expression evaluated using a chemiluminescence assay, two-three days after injection. Results are shown as mean +/- SEM. Experiments included at least 12-30 measurements with 3 different batches of oocytes. Significance was analyzed with one-way ANOVA follows by Holm-Sidak test. P<0.05 was considered significant.

No significant current was recorded with G424R, G424R, R438H and L439P mutants. Accordingly, as shown by the chemi-luminescence assay, these mutations were not addressed to the plasma membrane. By contrast, the L81P, A204T and R351P mutants produced currents lower by 40-70% as compared to wild-type CIC-Kb, but significantly different from non-injected oocytes (WT: 4.05±0.17μA, L81P: 1.47±0.19μA, A204T: 2.49±0.22μA, R351P: 2.84±0.29μA). Surface expression decreased in similar proportions. Finally, we investigated the effects of changing external Ca2+ from 1 to 10 mM on the currents produced by L81P, A204T and R351P mutants: a 45 to 70% increase was observed indicating that the sensitivity to external calcium, a characteristic property of CIC-Kb/Barttin, was preserved in mutants.

In conclusion, all CLCNKB mutations investigated in this study are pathogenic; the functional defects appear to be due to an altered targeting of the channel to the plasma membrane. Brochard K et al. Nephrol. Dial. Transplant. 31: 20-25 (2008).

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PC68

Estrogen induces internalization of KCNQ1 K+ channels in the colonic tumour cell line HT29c19A

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17β-estradiol (E2) rapidly reduces cAMP-dependent intestinal Cl− secretion by inhibiting K+ recycling (1). KCNQ1:KEC3 is the rate-limiting K+ channel involved in Cl− secretion in the intestine (2). Estrogen rapidly inhibits KCNQ1 channel current in the distal colon by a female sex-specific mechanism involving PKCδ and PKA-dependent phosphorylation of KCNQ1 (3, 4). The aim of this study was to determine if membrane trafficking plays a role in the E2 inhibition of KCNQ1 function in the human colonic cell line, HT29c19A. Data are given as Mean ± S.E.M. One-way ANOVA was used to establish the significance of the results.

Confocal immunofluorescence microscopy revealed that after 30 min E2 treatment (10 nM), the abundance of KCNQ1 protein remaining in the cell membrane (KCNQ1 remaining = 50.4%, n=3). As shown by biotinylation, E2 treatment halved the amount of KCNQ1 protein remaining in the cell membrane (KCNQ1 remaining = 50.4% ± 3.6% of control, n=5, P<0.001). The E2-induced removal of KCNQ1 from the cell membrane was partially reversed by rottlerin (KCNQ1 remaining = 75.6% ± 8.9%, n=5, P<0.05). Fluorescence co-localization studies indicated that E2-induced internalization of a subset of KCNQ1 channels into early endosomes, (early endosome marker, EEA-1; overlap coefficient with KCNQ1 = 0.30 ± 0.07 control, 0.60 ± 0.04 E2, n=4, P<0.05). Pre-treatment with rottlerin (5 μM, 30 min) completely reversed this effect (EEA-1; overlap coefficient with KCNQ1 was not different from untreated cells at 0.29 ± 0.06, n=4).

E2 treatment increased the phosphorylation of PKCδ by 56.2% ± 8.8%, n=6, P<0.001, within 5 min. This response was transient since no significant difference in PKCδ phosphorylation from control was observed after 30min E2 exposure (14% ±
8.5%, n=6). PKA activity was also increased after E2 treatment within 5 min (34.4% ± 7.5%, n=3 p<0.001) and remained activated after 30 min (33.9% ± 3.5%, n=3, p<0.001) with treatment; PKA associated with KCNQ1 within 5 min (2.1 ± 0.1 fold higher than control, n=3 p<0.01) and this association was prevented by pre-treatment with rottlerin (1.2 ± 0.3, n=3). E2 stimulated KCNQ1 phosphorylation (2.6 ± 0.9 fold higher compared to control, n=2) which was inhibited by rottlerin (1.08 ± 0.08, n=2). These results suggest that PKA:KCNQ1 association depends on prior PKCδ phosphorylation and leads to KCNQ1 phosphorylation. E2 rapidly induces retrieval of PKCΩ channel from the plasma membrane and promotes its internalization into an early endosome compartment. The internalisation of KCNQ1 is dependent on PKCδ phosphorylation and PKA:KCNQ1 association. O’Mahony F. et al. J. Biol. Chem. 2006; 282:24563-73. Preston P. et al. J Biol Chem. 2010; 5; 285(10):7165-75. Condiffe SB. et al. J Physiol. 2001 1; 530(Pt 1):47-54. O’Mahony F. et al. Mol Endocrinol 2009; 23(11):1885-99. This work was supported by NBIP Ireland and the Higher Education Authority (HEA) PRTLI Cycle 4

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PC69

Calcium- and voltage-dependence of the anoctamin2/TMEM16b-chloride channel: a candidate calcium-activated chloride channel in olfactory transduction

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Olfactory transduction in vertebrates involves a depolarizing chloride current activated by intracellular calcium. Indeed, the binding of odorants to receptors in the cilia of olfactory sensory neurons activates a transduction cascade via a cAMP-signal, which results in the opening of cyclic nucleotide-gated channels (CNG). The consequent Ca2+ influx through CNG channels produces an increased Ca2+ concentration in the cilia, which activates Ca2+-activated Cl- channels (CaCCs). Since olfactory sensory neurons maintain an unusually high intra-cellular Cl- concentration, CaCCs produce an efflux of Cl-. Therefore the Cl- current, contributing up to 90% of the total transduction current, amplifies the primary depolarization due to CNG current. Recent molecular and electrophysiological data strongly support that anoctamin2 (ano2, also called TMEM16b) is the molecular counterpart of CaCC in olfactory sensory neurons. Channel gating is both Ca2+- and voltage-dependent, but the mechanisms underlying the activation are still unknown. Ano2 does not have any apparent canonical Ca2+-binding site, but the first putative intracellular loop contains a series of negatively charged aminoacids. To test the hypothesis that this region is involved in channel activation, mutants of these residues were obtained and the functional properties of wild-type and mutant channels were measured and compared. After expression of wild-type and mutants of the mouse ano2 in a heterologous system (HEK293T cells), electrophysiological recordings with the patch-clamp technique in the inside-out configuration were performed. The exposure of the intracellular side of the channel to various Ca2+ concentrations rapidly activated a current both in wild-type and in mutant channels, indicating a direct activation by Ca2+. Currents showed a time-dependent decrease (inactivation) at -50 mV in the presence of a constant high Ca2+ concentration and an irreversible rundown. To determine the voltage dependence of the calcium sensitivity the wild-type and the mutant’s dose response relationships were compared. The Ca2+ concentrations for half-maximal current activation were: 4.6 ± 0.4 μM in the wild type and 6.5 ± 0.5 μM in one of the mutant at -50 mV, 2.8 ± 0.2 μM in the wild type and 4 ± 0.3 μM in the mutant at +50 mV (n=6, means ± s.d.). The Hill coefficients were between 1 and 2. The rectification properties of current-voltage relationships were calcium dependent both in wild type and in mutant channels with a strong inward rectification at high concentrations and an outward rectification at low Ca2+ concentrations. These preliminary results indicate that the region rich in negatively charged aminoacids, located in the first putative intracellular loop, could be involved in Ca2+- and voltage-dependence of the anoctamin2/TMEM16b-chloride channel.

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PC70

Regulation of Ion Transport in Cultured Human Bronchial Epithelial Cells by Cordyceps militaris Extract

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Cordyceps militaris (CM), also known as the caterpillar fungus, is a well-known traditional Chinese medicine. In recent decades, CM extract has been reported to have different biological activities, such as anti-tumor activity (Park et al., 2009) and immunomodulation (Shin et al., 2010). CM can be used to treat certain respiratory diseases, such as bronchitis and chronic obstructive pulmonary disease (Paterson, 2008). In this study, 16HBE14o-, a human bronchial epithelial cell line, was used as a model to study the regulation of ion transport by CM water extract (Yue et al. 2008). The 16HBE14o-cells were grown on Transwell-COL membranes until confluent (Wong et al., 2009). The monolayers were mounted in Ussing chambers, in which they were bathed in normal Krebs-Henseleit solution with a basolateral-to-apical Cl- gradient. An increase in short-circuit current (ISC) was measured by electrophysiological technique. Apical or basolateral application of CM extract stimulated a dose-dependent increase in ISC. The ISC responses were inhibited by apical pretreatment of 10 μM cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel blocker, CFTRinh-172: 2.92 ± 1.56 μA/cm2, n = 6, P < 0.05; Control (bl): 14.13 ± 1.56 μA/cm2. The apical membrane conductance regulator (CMEC) channel blocker, suppressed the increase in CM-induced ISC by 50.3% and 40.6% at the apical and basolateral sides, respectively. The apical ISC response was reduced by 38.1% after the basolateral application of 10 μM TRAM-34, an intermediate conductance Ca2+-activated K+ channel blocker. The basolateral application of chromanol 293B, a CFTR-activated K+ channel blocker, reduced the ISC response by 44.4%. However, both TRAM-34 and chromanol 293B did not significantly reduce ISC response stimulated by CM extract at the basolateral side. For-
thermoe, the CM extract-induced $I_{\text{CF}}$ could be significantly inhibited by adenylate cyclase (AC) inhibitor MDL-12033A, PKA inhibitor H-89, and intracellular Ca$^{2+}$ chelator BAPTA-AM. In conclusion, CM extract stimulated transepithelial Cl$^{-}$ secretion in 16HBE14o- cells through apical CFTR Cl$^{-}$ channels or CaCC. The basolateral camp- or Ca$^{2+}$-activated K$^+$ channels were activated by CM extract to provide a driving force for apical Cl$^{-}$ secretion. The underlying signal transduction mechanisms involve both AC/cAMP- and Ca$^{2+}$-dependent pathways.


This work was supported by a Direct Grant for Research from The Chinese University of Hong Kong (#2041539) and an RGC General Research Fund grant (#2140595). CM extract was provided by Professor KP Fung from the Institute of Chinese Medicine, The Chinese University of Hong Kong.

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PC71

Role of cysteine residues in the normal operation and assembly of K+-Cl- cotransporter isoform 2 (KCC2)

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The K+-Cl- cotransporters (KCCs) are polytopic membrane proteins that belong to the cation-Cl-cotransporter (CC) family. They are known to regulate intracellular K$^+$ and Cl$^{-}$ concentration as well as cell volume. Their transport activity is sensitive to N-ethylmaleimide (NEM), a thiol reagent that alkylates available sulfhydryl groups, and their C-terminus can interact with other CCCs to form oligomers. These features suggest that a number of cysteine residues within the KCCs play an important role in carrier activity and/or assembly. To determine whether this is the case, and which residues might be involved, we have exploited a mutagenic approach using rat KCC2 as a model. Cysteine residues substituted were those predicted to be cytoplasmic and, thus, belong to the N-or C-terminus. All cysteine-to-glycine substitutions (C8G, C742G, C854G and C982G) were generated by site-directed mutagenesis and heterologously expressed. In Xenopus laevis oocytes, two of the substitutions (C8G or C742G) produced no effects. However, C854G increased Rb$^+$ transport by 30.9% ± 0.1 and C982G decreased transport by 88.0% ± 2.0% (n=5) without altering cell surface protein expression as determined through biotinylation studies (n=2). They were therefore characterized in greater detail using the same expression system. Interestingly, they responded as did wild type (wt) carrier to the inhibitory effect of 250 $\mu$M furosemide (% decreases in activity were of 94.7 ± 0.3 for C854G, 91.4 ± 0.3 for C982G and 94.0 ± 0.4 for wt; n=3) but were more sensitive to the inhibitory effect 1 mM NEM (% decreases were of 32.8 ± 0.8 for C854G and 12.3 ± 0.3 for and C982G compared to 6.9 ± 1.1 for wt; n=3). To determine whether mutation-induced changes in NEM-sensitive transport activity could be due to changes in homologomeric assembly, communoprecipitation studies (n=2) were carried out using epitope-tagged carriers. Results showed that the quantity of carriers precipitated with wt KCC2 was lower for mutant C854G than for wt KCC2 or mutant C982G. Studies are underway to determine whether C854 and C982 are reactive to other thiol-specific reagents (such as MTSEA) and whether they can form disulphide bounds between each other or other residues. Lastly, FRAP studies in KCC2-transfected HEK-293 cells showed that NEM increased post-bleach fluorescence recovery time in a concentration-dependent manner (21.7 ± 1.1 sec compared to 15.2 ± 0.5 sec at 1 mM NEM). Among other possibilities, these results suggest that NEM decreases carrier mobility in cell membranes as would occur with higher order structures. Studies are also underway to determine the effect of C854G and C982G on fluorescent recovery time. Collectively, the data suggest that C854 and C982 play an important role in KCC2 function, perhaps by supporting homo- and/or heterooligomeric structure formation.

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PC72

TASK5: a study to determine its physiological role

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Two-pore domain (K2P) K$^+$ channels are active over physiological voltage ranges resulting in constituent leak of K$^+$ from the cell. These channels are fundamental in setting and regulating the resting membrane potential of cells, are regulated by physiological stimuli and play key roles in several physiological processes. To date fifteen members with distinct functional and pharmacological characteristics have been identified and grouped into distinct subgroups.

K2P15.1 (TASK5) was first identified in 2001 by two independent groups [1, 2] and although fails to show functional current in recombinant expression systems was included in the Acid Sensitive subgroup (TASK) of K2P channels primarily due to its sequence homology with other members of this group (K2P3.1 and K2P9.1). TASK channels are characterized by their sensitivity to external pH. At messenger level, K2P15.1 has a wide tissue distribution and shows high levels of expression specifically in pancreas and adrenal glands. While K2P15.1 is predicted to play an important role in both adrenal and pancreatic function, to date the physiological function and pharmacological profile of this channel has been elusive.

In this study, tissue distribution at messenger level of hK2P15.1 was determined by performing RT-PCR analysis of human fetal tissues. Subcellular localisation of hK2P15.1 was then examined by immunocytochemistry in HeLa cells transfected with hK2P15.1 and analysed by confocal imaging. Finally, hK2P15.1 interaction with specific proteins was investigated through in vitro pull-down assays using cytosolic domains of hK2P15.1 as bait and specific binding partners were identified by mass-spectrometry.

Transcript for hK2P15.1 was detected in human fetal kidney, eye, tongue, stomach and duodenum, but not in lung or muscle.

When transiently transfected in HeLa cells hK2P15.1 failed to show robust cell surface expression but appeared to be localized within the golgi and peri-nuclear areas. Candidate binding partners to hK2P15.1 have been identified and work is ongoing to verify the functional significance of these interactions.
Cerebrovascular Responses During Recovery From Hypercapnia in Young and Old Healthy Humans

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Cerebral vasodilator responses to progressive increases in hypercapnia are attenuated with age, diabetes, and hypertension (1, 2, 3). In addition, such vasodilator responses are reduced following cyclooxygenase (COX) inhibition (using indomethacin). Similarly, cerebral vasoconstrictor responses to progressive decreases in arterial CO2 (during the transition from hypercapnia to eucapnia) in young adults were blunted after indomethacin administration (4). However, it is unknown whether cerebral vasoconstrictor responses during this recovery are altered by aging, or whether COX inhibition alters this recovery response in older individuals. Twenty-one young (n=11; 26 ± 5y) and old (n=10; 65 ± 6y) healthy adults participated in the study. Subjects were fitted for a mask and breathed 6% CO2 for 3 minutes and then returned to breathing room air before (Pre) and 90 minutes after (Post) indomethacin administration (1.2mg/kg). Middle cerebral artery velocity (MCAv), cerebrovascular conductance index (CVCi), ventilation (VE) and end-tidal CO2 ($\text{ETCO}_2$) were averaged for each breath during recovery from hypercapnia. Values are means ± S.E.M., compared by a two-way repeated measures ANOVA. The change in MCAv during the transition from hypercapnia to eucapnia ($\Delta \text{MCAv}$) was greater in young compared with old subjects (36 ± 4 vs. 23 ± 3 cm/s; p<0.01) at Pre, and significantly blunted in both young (16 ± 2 cm/s) and old (11 ± 2 cm/s) by indomethacin. There were no group differences in the change in ETCO$_2$ (Δ $\text{ETCO}_2$) from hypercapnia to eucapnia, however, Δ $\text{ETCO}_2$ was greater after indomethacin in both young (17 ± 1 vs. 20 ± 1 mmHg; p<0.01) and old (15 ± 1 vs. 18 ± 1 mmHg; p<0.05) subjects. MCAv and CVCi responsiveness to CO2 during recovery from hypercapnia was not different between young and old subjects. However, MCAv responsiveness was reduced by indomethacin in both young and old adults (58% and 70% of Pre, respectively; p<0.001). There were no changes in VE sensitivity between groups or between Pre and Post. Taken together, this data demonstrates that cerebral vasoconstrictor responses to reductions in CO2 are preserved in healthy aging. In addition, COX inhibition blunted these vasoconstrictor responses, suggesting that prostaglandins have a role in regulating cerebrovascular tone in both young and old adults.


Physical training reduces time-domain ventilatory variability during exercise in healthy individuals

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Introduction: Ventilatory variability during exercise exhibits prognostic information in heart failure patients (1,2) and inversely correlates to left ventricle ejection fraction in heart failure patients (3). We have previously proposed the application of time-domain variability techniques to quantify ventilatory oscillations during exercise. Few studies have investigated strategies to reverse exercise periodic breathing. A recently published case report have shown reversion of exercise periodic breathing after 4 months of cardiac rehabilitation in a patient with heart failure (4). In a cross-sectional study, sedentary men exhibited higher ventilatory variability during maximal exercise test when compared to athletes (5). However, it is unknown whether this result is an effect of exercise training itself or a result from a selection bias. Objective: To evaluate the effects of physical training on time-domain variability during dynamic exercise in healthy subjects.

Methods: Randomized controlled trial where time-domain ventilatory variability during exercise was evaluated in 24 healthy individuals before and after 12 weeks of exercise training (Exercise group, n=12) or no intervention (control group, n=12). Standard deviation (SD) and root mean square successive difference (RMSSD) of VE, RR and Vt during exercise test were calculated for each patient, and normalized by the number of respiratory cycles (SD/n and RMSSD/n, respectively) (5).

Results: Before training, the groups were comparable. After 12 weeks, volunteers in the exercise group presented higher peak oxygen consumption (37.12±2.51 vs. 25.9±1.46, P<0.001) and lower body mass index (23.39±0.70 vs. 26.32±0.70, P=0.008) than individuals in control group. In addition, time-domain variability of respiratory rate during exercise was higher in the control group when compared to volunteers in the exercise group after intervention (table 1). Conclusion: Exercise training reduced time-domain ventilatory variability of healthy individuals during a maximal exercise test. The impact of this adaptation remains to be evaluated.
Oxygen uptake kinetics during moderate intensity cycling in obese individuals
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Peak oxygen uptake (VO2peak) is reduced in obese individuals in comparison with lean individuals. In addition to the dynamic response of oxygen uptake during the initial period of exercise, it is slowed in obese adolescents during sub-maximal cycle exercise at specific percentages of VO2peak. However, as yet no studies have examined VO2 kinetics responses during exercise intensities performed relative to ventilatory threshold (VT) and/or in obese adults. Accordingly the purpose of this study was to investigate the VO2 kinetic response during moderate intensity cycle exercise performed relative to VT in obese participants. To explore underlying mechanisms of potential differences, cardiac output (CO) responses were also measured. Fourteen obese (mean±SD, age: 55.1±5.8 yr, BMI: 32.1±1.7 kg.m-2) (7 men, 7 women) and fourteen control (age: 50.8±5.8 yr, BMI: 23.3±1.6 kg.m-2) (7 men, 7 women) individuals completed a graded cycle test to volitional exhaustion for the determination of VO2peak. On a separate occasion subjects completed four 6-min bouts of constant load cycle exercise at 80% VT for the determination of VO2 kinetics. The dynamics of the VO2 response were established by fitting a bi-exponential function to the VO2 data (averaged from 4 bouts). An indicator of the rate of change for the total VO2 response was obtained by calculating the mean response time (MRT), which is the time taken to reach 63% of the total amplitude from baseline to end-exercise. CO was measured at rest and during exercise at two time points (30 s and 240 s) during two further 6-min bouts using a closed circuit inert gas rebreathing technique. Values are means ± SD compared using an unpaired Student’s t-test.

Peak VO2 was significantly lower in obese (24.1±5.2) than control (30.6±8.0) subjects (P<0.05) but VO2peak (ml.kg-1.m-1) at VT was similar between obese (18.3±5.8) and controls (22.3±6.6). There were no differences between obese and control subjects in the time constant of phase II (s) of the VO2 kinetic response (40.4±11.2 vs. 36.6±10.0 respectively), MRT (s) (50.3±9.9 vs. 46.5±10.8 respectively) or the time constant (s) of the heart rate kinetic response (63.7±24.8 vs. 58.7±10.6 respectively. Similar responses were observed between obese and control groups in relation to CO (L.min-1) during exercise at 30 s (9.4±2.8 vs. 9.1±2.7, respectively) and 240 s (11.1±3.0 vs. 11.4±3.2 respectively). In addition the rate of adaptation in CO from rest to steady state exercise (% steady state CO) was not different between obese (70.0±14.4) and control (62.4±13.5) groups.

The results suggest that despite a lower maximal aerobic capacity, sub-maximal exercise performance in grade 1 obesity (range of BMI: 30-35) seems to be preserved. This was shown by similar VO2 responses at VT and similar dynamic responses of VO2 and CO during moderate cycle exercise in obese and lean groups.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC75

Brain blood flow and cardiovascular responses to hot flashes in symptomatic postmenopausal women
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Hot flashes can significantly reduce blood pressure in some symptomatic women. However, it is unknown whether this drop in blood pressure compromises cerebral perfusion. Objective: This study tested the hypothesis that cerebral perfusion is reduced during the postmenopausal hot flash. Method: Eleven healthy, normotensive, postmenopausal women rested in a temperature-controlled laboratory (25 °C) for approximately 120 minutes while waiting for a hot flash to occur. The onset of a hot flash was objectively identified by an increase in skin blood flow (laser Doppler flowmetry) and/or sternal sweat rate (capacitance hygrometry). Middle cerebral artery blood velocity (MCAvmean, transcranial Doppler), mean arterial blood pressure (MAP, Finometer®), systolic aortic blood velocity (Doppler of ascending aorta, an index of stroke volume) and heart rate (HR, electrocardiogram) were measured continuously. Cerebrovascular resistance (CVR) was calculated as MCAvmean/MAP. Data were averaged into 10 s segments to identify the maximum drop in MAP during the hot flash. For each hot flash, all physiological responses at the period of the maximal drop in MAP were compared to the resting baseline value taken immediately prior to a hot flash. Results: Twenty-seven hot flashes occurred during the experimental sessions (lasting 6.2±2.8 min, 3±1 hot flashes per participant). The average maximum decrease in MAP during the hot flashes was 12±5 mm Hg (P <0.01). At the maximum
decrease in MAP, there was a modest reduction in both MCAvmean (4.7 ± 1 cm/s, P = 0.01) and CVR (0.1 ± 0.3, P = 0.01), while systolic arterial blood velocity and HR did not change (P > 0.05 for both). Conclusion: A hot flush can be accompanied by a significant acute reduction in MAP in symptomatic postmenopausal women. However, this response corresponded to a small decrease (i.e., ± 6 %) in cerebral perfusion as measured by MCAvmean. Thus, hot flushes only mildly perturb cerebral perfusion.

Supported by NIH Grant AG030189

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PC77

MCα and MCγ activation inhibits caspase-3 production and activity, promotes cell viability, IL-10 and HO-1 release from human C20/A4 chondrocytes

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Chondrocyte cell death due to inflammation is an important risk factor predisposing to osteoarthritis and exacerbating the disease progression1,2. Here, we report protective effect of the melanocortin receptor (MC) pan-agonist αMSH and the selective MCα agonist DTRPγ-MSH against TNF-α-induced apoptosis2 in human C20/A4 chondrocytes as assessed by determination of caspase-3 levels, caspase-3/7 activity and cell viability using MTT assay. We show that melanocortins significantly increase the anti-inflammatory proteins heme oxygenase (HO)-1 and interleukin (IL)-10. We show that melanocortins significantly increase the anti-inflammatory proteins heme oxygenase (HO)-1 and interleukin (IL)-10. C20/A4 chondrocytes were plated at 1.5x10^6 cells/cm² and treated with PBS, TNF-α, and DTRPα-MSH prevented TNF-α-induced cell death, causing an inhibition of caspase-3/7 activity and an increase in IL-10 and HO-1 production. These results indicate a protective role of these peptides against TNF-α-induced chondrocyte apoptosis and induction of anti-inflammatory proteins and thus could represent a promising novel approach for treatment of OA.


MK is supported by Research Committee, University of Westminster. (Grant Code C planetary); Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC78

Distinct effect of EPA and DHA on anabolic signaling pathways in C2C12 skeletal muscle cells

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The combination of Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) supplementation, two main types of Long-chain n-3 polyunsaturated fatty acids (LCn-3 PUFAs) found in fish oil, has previously been shown to increase protein anabolism in both young animal models (Gingras et al, 2007) and elderly humans (Smith et al, 2011) via the mTOR-p70s6k pathway. Individually EPA has been reported to reduce the effect of proteolytic factor (PIF) on protein degradation in C2C12 (Smith et al, 1999) and has also been found to attenuate the deleterious effects of TNF-alpha during skeletal muscle C2C12 differentiation (Magee et al, 2007). However, research into the distinct contribution of EPA and DHA to protein anabolism, are at present, limited. The present study aims to clarify the distinct effects of EPA, DHA and a mixture of EPA and DHA on anabolic signaling pathways in C2C12 myocytes. On differentiation day 4, C2C12 cells were incubated with differentiation medium containing either EPA (50 μM), DHA (50 μM), a mixture of EPA (50 μM) and DHA (50 μM) or control for 24 h. Cells were serum starved for 4 h, followed by amino acid deprivation period for 1 h and then stimulated with 2mM L-Leucine for 30 min. Phosphorylated and total amounts of Akt[Ser473], mTOR[Ser2448], 4EBP1[Thr37/46], p70s6k[Thr389] and rps6[Ser235/236] were measured by western blotting. Data were analysed by one-way ANOVA and independent t-tests. Data are expressed as mean ± S.E.M. EPA, DHA, and a heterogeneous mixture of EPA and DHA produced a similar increase (P<0.05) in p70s6k[Thr389] phosphorylation, a key protein in the initiation step of protein translation, compared to control condition. This increase was 14.8 ± 5.3 % for EPA, 24.6 ± 8.2 % for DHA, and 27.9 ± 11.2 % for a mixture of EPA and DHA with no change in phosphorylation of Akt[Ser473] and mTOR[Ser2448]. The phosphorylation of 4EBP1[Thr37/46] was increased to 84.3 ± 39.4 % from the control condition (P<0.04) in response to EPA stimulation but not in that of the other treatments. This study has demonstrated that EPA, DHA and a mixture of EPA and DHA can stimulate p70s6k activation. Interestingly, only EPA can activate the phosphorylation of 4EBP1 in C2C12 cells.

Yap is a regulator of satellite cell fate.

and self-renewal and are consistent with the hypothesis that Ser127 change during satellite cell activation, differentiation and self-renewal, myogenin+ than self-renewing, Pax7+ satellite cell progeny. Phospho-Yap Ser127, however, appeared in clusters that contain inhibitory Yap Ser127 phosphorylation changes during satellite cell activation, proliferation, differentiation and self-renewal.

Mouse muscle fibres were isolated with their satellite cells retained in their niche using an established protocol (Collins & Zammit, 2009). Fibres were then cultured in suspension, fixed at 0, 24, 48 or 72 h post isolation and immunostained for myogenic markers (MyoD) or differentiating satellite cells (myogenin).

Resting +Gz tolerance (RGT) was assessed by reference to each participant’s subjective point of visual ‘grey-out’ prior to each exposure set. Respiratory mass spectrometry and a mixing box/tracer gas technique were used to measure VO2 and expired CO2 (VCO2). Heart rate (HR) was also measured.

RGT improved in FC-AGT compared with PC-AGT (5.9 ± 1.2 vs 5.3 ± 0.6 +Gz, P < 0.05). ANOVA revealed a significant effect of AGT type and +Gz level on VO2, VCO2 and HR, with a significant +Gz level interaction observed for VO2 (P < 0.01) but not VCO2 (P = 0.09) or HR (P = 0.23). In PC-AGT, VO2, VCO2, and HR increased from baseline to peak at 1.4 ± 0.3 l·min⁻¹ and 166 ± 11 bpm respectively at +8Gz. At +6Gz, FC-AGT significantly reduced the increase in VO2 (25%), VCO2 (26%) and HR (18%). There was a similar but less pronounced effect at +7Gz (VO2 [15%], VCO2 [15%] and HR [19%]). At +8Gz, only the increase in HR was significantly attenuated with FC-AGT (15%). During the SACM, VO2 (17%), VCO2 (25%) and HR (23%) were lower (P < 0.05) compared with PC-AGT. VO2 and HR were correlated with PC-AGT (Spearman’s, ρ=0.86, P < 0.05) but not FC-AGT (ρ=0.44, P = 0.07). Thus, FC-AGT improved RGT and attenuated the energy cost of resisting the effects of increased +Gz, notably above +6 Gz. The reduced energy cost in FC-AGT may delay the onset of fatigue in aircrew during repeated AGSMs and reduce the risk of G-LOC. Hence, FC-AGT might be more appropriate in aircraft capable of sustaining high (> +6 Gz) accelerations. The different relationships between VO2 and HR may reflect alternate mechanisms operating to increase HR; HR principally increased through baroreceptor activation in FC-AGT and through exercise in PC-AGT.

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Effect of Momordica charantia on Blood Cholesterol level in Albino Rats

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Momordica charantia, commonly known as bitter gourd, is used as a vegetable by the Asian community in Africa. It is frequently used as an anti-diabetic herb for the management of disease in the Ayurvedic system of Medicine. This present study was aimed at evaluating the effects of M. charantia on blood cholesterol levels in albino rats.

The study involved 25 rats and they were divided into 5 groups each comprising of 5 rats. The aqueous extract of Momordica Charantia was administered orally with syringes and cannula to 4 groups at different doses (80mg/kg, 100mg/kg, 120mg/kg and 140mg/kg body weights per day, respectively) and the last group served as the control and were given drug vehicle (normal saline) only. After two weeks of administration, the 25 rats were sacrificed and blood samples were collected and assayed for blood cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein levels.

Results indicated that M. charantia plant extract increased significantly (P<0.05) the blood cholesterol level and density lipoprotein levels in the experimental group 3 (100mg/kg), and significantly reduced low density lipoprotein levels (P<0.05) in the experimental group 2 (80mg/kg), when compared to the control group.

This study showed that M. charantia plant extract has blood cholesterol modifying effects and these effects were probably dose-dependent.

Total Cholesterol In Control And Experimental Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Total Cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1</td>
</tr>
<tr>
<td>Group 2</td>
<td>3.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.9</td>
</tr>
<tr>
<td>Group 4</td>
<td>2.6</td>
</tr>
<tr>
<td>Group 5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

LDL-C in control and experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean LDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.4</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.3</td>
</tr>
<tr>
<td>Group 5</td>
<td>1.2</td>
</tr>
</tbody>
</table>


Dynamic cerebral autoregulation remains preserved in professional boxers

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Background and hypothesis: Chronic traumatic encephalopathy (CTE) associated with boxing is characterised by progressive impairment in cognitive, behavioral and motor function as a consequence of repetitive impact to the brain. Given that symptoms typically present long after the cessation of a boxer’s career, early diagnosis has the potential to reduce the risk of developing CTE. Traditionally, much emphasis has focused on neuro-anatomical correlates (McCrory et al., 2007) to the exclusion of potential abnormalities in cerebral haemodynamic function. In the current study, we tested the hypothesis that dynamic cerebral autoregulation (dCA) would be impaired in boxers and thus provide a more sensitive diagnostic for CTE.

Methods: Eight currently active professional male boxers aged 28 (mean) ± (SD) 6 years with clinical signs of mild CTE were compared to 12 activity-matched (non-boxer) controls (29 ± 5 years). The boxers included World, British and Commonwealth champions who had boxed 137 ± 97 professional rounds over 4-19 years. All boxers had a prior history of loss by technical or total knock-out. Transfer function analysis (TFA) of spontaneous oscillations in mean arterial pressure (MAP, finger photoplethysmography) and middle cerebral artery blood flow velocity (MCAv, trans-cranial Doppler ultrasound) in the low frequency (LF) range (0.07-0.20 Hz) was employed for the measurement of dCA (Zhang et al., 1998). Cerebrovascular resistance (CVR) was calculated as MAP/MCAv and cerebrovascular conductance (CVC) as MCAv/MAP. Following confirmation of distribution normality using Shapiro-Wilk W tests, data were analysed using independent samples t-tests.

Results: The data presented in Table 1 identified that all aspects of cerebral haemodynamic function in boxers was comparable to that observed in controls with no evidence of any impairment. Furthermore, no differences were observed in TFA in either the very low and high frequency ranges.

Conclusion: These findings are the first to demonstrate that dCA remained preserved in boxers in spite of on-going repetitive head trauma thus excluding its suitability as a haemodynamic risk factor for CTE.

Table 1. Cerebral haemodynamic function

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=12)</th>
<th>Boxers (n=12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>94.9 ± 9</td>
<td>93 ± 8</td>
<td>0.65</td>
</tr>
<tr>
<td>CVR (mm Hg/mmHg)</td>
<td>1.41 ± 0.37</td>
<td>1.76 ± 0.57</td>
<td>0.86</td>
</tr>
<tr>
<td>CVC (mm Hg/mmHg)</td>
<td>0.98 ± 0.17</td>
<td>1.64 ± 0.17</td>
<td>0.76</td>
</tr>
<tr>
<td>LF MCAv Power ( Units)</td>
<td>5.23 ± 1.32</td>
<td>5.29 ± 1.97</td>
<td>0.52</td>
</tr>
<tr>
<td>LF MAP Power (Units)</td>
<td>5.68 ± 2.58</td>
<td>5.43 ± 2.48</td>
<td>0.72</td>
</tr>
<tr>
<td>LF CVC Power (Units)</td>
<td>3.51 ± 1.09</td>
<td>3.05 ± 0.92</td>
<td>0.31</td>
</tr>
<tr>
<td>LF Phase (Radians)</td>
<td>0.64 ± 0.26</td>
<td>0.57 ± 0.25</td>
<td>0.66</td>
</tr>
<tr>
<td>LF Cerebrovascular (Units)</td>
<td>0.03 ± 0.05</td>
<td>0.04 ± 0.11</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values are mean ± SD


This study was supported by a JPR Williams Research Fellowship
PC83
Effects of immobilization and rehabilitation on human lower leg muscles measured using magnetic resonance imaging
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Muscle atrophy occurs rapidly during cast immobilization, rehabilitation is a clinical challenge and the time course of changes during these phases is poorly understood. The aim of this study was to use magnetic resonance imaging (MRI) to generate imaging biomarkers of muscle function and we report here the changes occurring during 8 weeks of rehabilitation following 6 weeks of cast immobilization.

Eighteen patients (8 M, 10 F), who had one lower leg (13 right, 5 left) immobilized in a cast were reported previously (1). Seventeen completed a rehabilitation phase comprising a strength training regime with strength assessment (KinCom dynamometer) and MRI (Philips Achieva 3.0 T) at two-weekly intervals. The same protocol was followed by 44 volunteers in 3 age groups (20-30yrs, 7 M, 9 F; 50-65yrs, 7 M, 7 F; and >70 yrs, 8 M, 6 F). Cross-sectional area (CSA) was measured for tibialis anterior (TA), gastrocnemius (Gast) and soleus (Sol) from high-resolution T1-weighted spin-echo images. Volume was calculated from the product of CSA and the distance from the tibial plateau to the insertion of Gast into the Achilles tendon. Penetration angle was measured in Gast from a high resolution T1-weighted spin-echo image. Fractional fat/water ratios were calculated using a 3-point Dixon method. T2 relaxation time before and after exercise was determined using a multi spin-echo scan. Measurements other than volume were taken at a point 70% of the distance from the lateral malleolus to the tibial tuberosity.

In patients, strength increased by 3.4 N/day in plantarflexion and 1.1 N/day in dorsiflexion. CSA largely recovered to baseline values apart from Sol which was still ~5% smaller than the contra-lateral leg at day 99. T2 peaked at the end of the cast period for TA and Sol but continued to rise until day 57 for Gast before returning to baseline. In the contra-lateral leg T2 was unchanged. Penetration angle decreased during immobilization but recovered rapidly following cast removal. In volunteers, strength increased by 2.8 N/day in plantarflexion and 0.4 N/day in dorsiflexion. No changes were found in any MRI measures. T2 in each muscle increased with age and in Gast showed the largest response to exercise.

In healthy volunteers, exercise had no effect on any of the MRI measurements, despite an increase in strength. Strength increases in patients were slightly larger than volunteers and were reflected in changes in T2. The increase in T2 with age in the volunteers is in the same direction as that induced by atrophy, although not so marked. T2 may, therefore, provide an imaging marker for atrophy and sarcopenia.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC84
Effects of an Inverted Body Position on Arm Maximal Voluntary Contract Force and Cardiovascular Parameters
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1Kinesiology & Health Studies, University of Regina, Regina, SK, Canada and 2School of Human Kinetics & Recreation, Memorial University, St John’s, NF, Canada

Some recent research has suggested that differences in maximal voluntary contraction (MVC) in an upright vs. inverted postural position are related to alterations in sympathetic nerve stimulation, which could alter cardiovascular responses (Paddock & Behm 2009). Our purpose was to determine the effects of postural changes on cardiovascular responses during upper body (arm) MVC. We hypothesized that inversion would elicit a significantly different effect on selected cardiovascular parameters. Twelve healthy male human subjects (age = 22.5 ± 1.6yr; mass= 82.1 ± 17.1kg; BMI= 22.3 ± 3.5) completed three postural trials in random order on separate days: upright seated (U), supine seated (S), and inverted seated (I) position in a specially designed inversion chair. At baseline, a five-second elbow flexion MVC was performed with the elbow positioned at 90 degrees. The subject was then positioned for 150 sec in each posture, followed by a 30sec MVC (MVC30). During each trial, stroke volume (SV), cardiac output (Q), heart rate (HR) and mean arterial blood pressure (MAP) measurements were recorded using continuous monitoring finger plethysmography (Finometer). MVC force (N) was averaged over the 30sec. The ANOVA results showed statistical differences in MVC force from baseline to post MVC30 for all postures (U=190.9 ± 55.7N vs. 154.2 ± 57.5N; S=191.7 ± 94.0N vs. 167.2 ± 73.9; I=176.7 ± 42.1N vs. 158.2 ± 37.5), but no significant force differences between postures at MVC30. Statistical differences were found for the cardiovascular variables HR, SV, and MAP from baseline to MVC30. Only HR and Q were significantly different between postures for U vs. S (HR=15.7%; Q=7.6%) and U vs. I (HR=15.6%; Q=7.6%). Collectively, the cardiovascular results presented here suggest that the significant reduction in HR and Q between postures may be related to an inversion-induced inhibition of sympathetic stimulation, supporting previous research (Bosone et al. 2004; Hearn et al., 2009). Further research is warranted to investigate whether prolonged inversion has an effect on neuromuscular and cardiovascular parameters. This has important implications for scenarios where individuals may have to perform muscle contractions under inverted conditions (e.g., overturned submerged helicopter or motor vehicle, military operations).


We wish to thank all participants involved in this study. Financial support was provided by grants from NSERC Canada (JPN, DGB).
The decrease of FiO₂ was not a limiting factor of endurance at VO₂ max

H. Petot, R. Meilland, M. Landrain and V. Billat

UBIAE U902 INSERM, University d’Evry Val d’Essonne, Evry, France

VO₂max and endurance at VO₂max are currently used as markers for training prescription and to evaluate the physical fitness and health. If the limiting factors of VO₂max are well understood as is the effect of acute hypoxia, then the limiting factors of endurance at VO₂max need to be clarified. Thus, the purpose of this study was to demonstrate that 1) hypoxia decrease the VO₂max plateau amplitude but not the VO₂max plateau duration 2) was caused by the fact that the first power elicited VO₂max (PVO₂max) was decreased by hypoxia and consequently this decrease of power output in hypoxia allowed to maintain the same VO₂max plateau duration than in normoxia. Ten subjects performed four cycling tests at three days interval: 1) Two incrementals tests in normoxia (IncN) (FiO₂ = 20.93 %) and in acute hypoxia (FiO₂ = 14.5 %) to determine VO₂max, the power at VO₂max (PVO₂max) and the lactate threshold (PLT) in both conditions 2) two time limit (tlim) at VO₂max in both condition in which the power was decreased just after the subject reached VO₂max to determine the endurance at VO₂max (tlim@VO₂max). The result showed that tlim@VO₂max were not significantly different between the two conditions (702.5 ± 572.5 s in N vs. 869.5 ± 782.5 s in H, p = 0.18) whereas VO₂max decreased by 9.6 ± 5.6 % and PVO₂max decreased by 17 % in hypoxia. tlim@VO₂max in hypoxia was correlated with tlim@VO₂max in normoxia (p = 0.0004 with r = 0.9). tlim@VO₂max was correlated with the number of power waves during the plateau (p = 0.007 with r = 0.78) but not with the delta VO₂max (p = 0.94), the delta PVO₂max (p = 0.69) or the delta of oxygen blood desaturation (p =0.58) between N and H. In conclusion, the study showed that the decrease of FiO₂ was a limiting factor of the VO₂max amplitude but not of the endurance at VO₂max and that the endurance at VO₂max in hypoxia depend on the power variation during the VO₂max plateau but not on the subjects physical fitness.

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Assessment of the efficiency of two high-altitude walking strategies in humans

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Surveys of risk factors for acute mountain sickness (AMS) in trekkers report height gained as important (1), which is usually interpreted as the effect of the fall in the ambient partial pressure of oxygen. However, it could equally be due to or compounded by the effect of greater exercise required in achieving a higher altitude. Exercise at high-altitude has been linked with an increased risk of AMS (2) and is performed intermittently when the intensity is high or when working capacity is reduced (e.g. at high-altitude (3)). However, to-date the efficiency of exercise/work at high altitude has not been documented. Work efficiency, calculated from the total oxygen cost (VO₂) of work, is lower in intermittent compared with continuous exercise, achieving the same average power output (4). We hypothesised that similar, but exaggerated results will be found at altitude and that perceived exertion would be greater with intermittent exertion (5). Our study assessed the efficiency of two trekking strategies, a steady continuous walk (CW) and an intermittent high-speed walk with rest (IW), when completed in hypoxia. Nine high altitude naive volunteers (age: 21 ± 1 years; height 170 ± 12 cm, body mass: 68.3 ± 15.0 kg; hypoxia-specific peak uphill walking speed (PWS) 2.1 ± 0.2 m/s¹) completed two uphill (10%) walking sessions on a powered treadmill, following a crossover design, in a normobaric hypoxic chamber (simulated altitude 3500 m; O₂ 13.5%; CO₂ 0.04%; N₂ balance; ambient temperature 10°C; relative humidity 20%); 1) CW: 30 min walk at 50% PWS, 2) IW: a timed walk to complete CW distance at PWS, with participants resting as they required. Breath-by-breath VO₂ was measured concurrently with heart rate (HR) and arterial oxygen saturation (SpO₂). Ratings of perceived exertion (RPE) and breathlessness were taken each min and blood lactate measured 3-min post-walk. Data were analysed with paired t-tests and Wilcoxon signed-ranks tests as appropriate. The mean total work was 124 ± 37 kJ for which there was no difference in the time to complete between conditions (CW vs. IW: 1800 ± 1729 ± 291 s). Total VO₂ was greater for the IW condition (3431.1 ± 1551.9 vs. 5024.9 ± 16361 L, p < 0.05) as were peak and mean HR (peak: 168 ± 24 vs. 192 ± 13; mean: 129 ± 16 vs. 161 ± 11 b/min¹, p < 0.01), but no difference in mean or minimum recorded SpO₂. Ratings of perceived exertion and breathlessness were higher for the IW condition (RPE: 10 ± 1 vs. 17 ± 1; breathlessness: 2 ± 1 vs. 5 ± 1, p < 0.01), as was blood lactate (1.23 ± 0.47 vs. 7.15 ± 1.13 mmol/L¹, p < 0.01). The decreased efficiency and greater RPE for the IW condition in hypoxia agree with those findings reported by others for normoxia (4, 5). Importantly our findings indicate that a steady continuous walk is the most efficient strategy to adopt when high-altitude trekking and may reduce the risk of AMS.

Basyet B et al. (1999). Aviat Space Environ Med 70, 867-873.

Performance and stroke volume during marathon in recreational middle-aged runners

V. Billat1, H. Petot1, M. Landrain1, R. Meilland1, J. Koralsztein2 and L. Mille-Hamard1

1UBIAE U902 INSERM, University d’Evry Val d’Essonne, Evry, France and 2Sport Medicine Center CCAS, Paris, France

Background: Despite the increasing popularity of marathon running in recreational athletes and scientific research on phys-
ioLOGICAL PERFORMANCE DURING RACES, NO INFORMATION IS AVAILABLE ON THE STROKE VOLUME AND CARDIAC OUTPUT RESPONSES TO EXERCISE.

Methodology/Principal Findings: To test the hypothesis that: 1) stroke volume, heart rate and cardiac output values during a marathon race are in sub maximal steady states while speed decreases and; 2) cardiac endurance i.e. the ability to run at a high fraction of the speed at the maximal stroke volume and the upward drift of the cardiac output/speed ratio could be respectively positively and inversely related with the performance (i.e. mean speed over the race), we measured the stroke volume (SV), heart rate (HR) and running speed of 14 recreational runners (3h30min±45min) in an incremental maximal test and during a real marathon race. Results showed: 1) that HR, SV and cardiac output (CO) were in steady state at high but submaximal values (87.0±1.6%, 77.2±2.6%, and 68.7±2.8% of maximal values of HR, SV and CO respectively). In addition, the cardiac steady state was in response to a constant speed linear decrease after the 14th kilometer from 81.1±7.3 to 66.8±10.2 % of the speed at the maximal oxygen uptake determined in the incremental test (r=0.94; p<0.001); 2) marathon performance, was inversely correlated with this upward drift of the cardiac-output/speed ratio (ml of CO/m-1) (r=0.65, p<0.01) and positively related to the ability of the runner to complete the marathon at a high percentage of the speed at the maximal stroke volume (r=0.83, p<0.0002).

Conclusion/significance: Overall, the results show that speed at the maximal stroke volume (r=0.83, p<0.0002). Runner to complete the marathon at a high percentage of the speed at the maximal stroke volume (r=0.83, p<0.0002) and positively related to the ability of the runner to complete the marathon at a high percentage of the speed at the maximal stroke volume (r=0.83, p<0.0002). In association with VO2max, these factors could be considered to be classical determinants of performance in the global endurance and energy cost in marathon running.

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**PC88**

**Do physiological time delays provide the reason for intermittent, visual-manual control?**

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It has been almost exclusively assumed that a continuous paradigm from engineering control theory is most suitable in explaining sustained control of human movements. However, recently, it has been shown that controlling an unstable load serially ballistically through intermittent taps applied to a joystick is natural and more effective than using continuous hand contact (Loram et al., 2011). Furthermore, it was found that, when maximizing positional control, participants adopted a Median Tapping Interval (MTI) of 0.450 (0.2 iqr) which corresponds to two taps/s. However, the physiological basis of this interval is unknown. Here we studied whether the MTI is determined by intrinsic physiological factors (such as the feedback time delay) or by external load dependent factors such as the complexity (order) or stability of the load being controlled.

In ten different conditions ten participants, using a joystick, controlled the position of a dot (representing the position of a virtual load running in real time) on an oscilloscope for 200s. Participants were asked to keep the load position close to the centre of the screen by keeping either continuous or intermittent contact with the joystick. In eight trials the joystick position modulated the acceleration of the load (2nd order) whereas in the remaining two trials it specified the velocity of the load (1st order).

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an auto regressive moving average (ARMA) process. Subsequently, it was assessed to what value we had to build up the interval between the first and second stimulus in order to make the second response time identical to the first. In other words, the RP equals the inter-stimulus interval for which the reaction time to the first stimulus matches the reaction time to the second stimulus.

Our method showed that in a zero order pursuit task, participants were refractory to the second stimulus for, on average, 0.284 (0.05 sd) s.

Refractoriness is problematic for any linear time invariant model trying to fit human behavior. Intermittent open loop control, on the other hand, is an appropriate solution to refractoriness (and online time delays in general (cf. Gawthrop et al., 2011)). A refractory period of this size has been predicted to explain the upper frequency limit of significant coherence (the control bandwidth) in visual manual compensatory tracking (Loram et al., 2011, J. Physiol). Questions arising from this preliminary finding and previous research (Loram et al., 2009) are: 1) does this psychological refractory period generalize to other tasks? 2) Will we find the same values when varying the complexity (order) or stability of the load being controlled? 3) And is the refractory period related to the physiological visual feedback time delay in humans?


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**PC90**

**Optimal control of task-specific muscle synergies I: Feedforward strategies**

K. Nazarpour and A. Jackson

Newcastle University, Newcastle, UK

Are muscle synergies hard-wired into neural circuits that reflect conserved biomechanical constraints [1] or can they be optimised to suit abstract task requirements [2]? We addressed this question using a myoelectric-controlled interface [3] which dissociates task requirements from limb biomechanics. 16 human subjects made repeated movements of a myoelectric cursor controlled by smoothed, rectified electromyogram (EMG) from two muscles. Elliptical target shapes imposed relevant and irrelevant dimensions within the task space, oriented such that movement variability should optimally be constrained along dimensions of either positive or negative EMG covariance. Muscle combinations included natural synergists (FDI-APB, ECR-FCR) and unnatural pairs (FDI-ADM, ECR-APB). Performance improved consistently over recording blocks, although scores were slightly lower for unnatural muscle pairs. An index of covariation (IoC) quantified the trial-to-trial cursor variability along axes of positive and negative EMG covariation. Multiple regression analysis of IoC revealed the main effect of target orientation for all the pairs, consistent with a buffering of movement variability into the appropriate task-irrelevant dimension. For natural synergists, the interaction between target shape and block number indicated a significant effect of training. The influence of target shape on IoC increased through the hold period consistent with a minimum intervention controller acting on visual feedback. However, even in the absence of the visual feedback, variability patterns were still modulated by target orientation although overall IoC values were shifted in the direction of positive covariation. A simple feed-forward model was used to predict optimal distributions of activity across a many-to-two cortico-motoneuronal projection in the presence of signal-dependent noise. The model explained (1) target-dependent modulation of IoC and (2) the overall positive covariation in the absence of visual feedback. A further prediction that common drive to muscles should vary with target orientation was verified by intermuscular coherence analysis. Enhanced beta-band coherence between FDI-APB was observed for targets that required positive EMG covariance.

Rather than being limited to a small number of fixed synergies, the human hand can recruit a wide repertoire of co-ordinated muscle patterns appropriate for task demands. Convergence and divergence in cortico-spinal projections to distal motoneurons provide a rich neural substrate for flexible feedforward minimisation of movement errors in task-relevant dimensions. In the presence of feedback, this strategy is enhanced by incorporation of minimum intervention control. Mussa-Ivaldi, F.A., and Bizzi, E. (2000). Motor learning through the combination of primitives. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355, 1755-1769.


MRC and Wellcome Trust

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC91**

**Optimal control of task-specific muscle synergies II: Feedback strategies**

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A companion abstract [1] reported the development of optimal correlation structure in the trial-to-trial variability of muscle activity according to the abstract demands of a myoelectric-controlled interface task [2]. One mechanism by which this can emerge is through modulation of the common drive to motoneurons via divergent corticospinal projections. However, this simple feed-forward model, while accounting for behaviour in the absence of visual feedback, fails to describe several features of the data when visual feedback is present. In particular, the observed negative correlation between muscles implies the action of a feedback mechanism acting predominantly along task-relevant dimensions. In this experiment we sought direct evidence of such a ‘minimum intervention controller’ [3] driven by either visual or proprioceptive feedback. 20 human subjects made repeated movements of a myoelectric cursor controlled by smoothed, rectified electromyogram (EMG) from APB and ADM muscles. Elliptical target shapes imposed task-relevant and irrelevant dimensions within the task space, oriented such that movement variability should optimally be constrained along dimensions of either positive or negative EMG covariance. In half of the trials, a perturbation was delivered 300ms into the 1s hold period. Perturba-
tions were either visual (cursor position was translated along the axis of one of the controlling muscles) or proprioceptive (electrical stimulation of median nerve at the wrist or cutaneous nerve of the little finger in the absence of visual feedback).

The existence of a minimum intervention controller acting along the task-relevant dimension predicts that reflex responses should be observed in the unperturbed muscle. Furthermore, these responses should change direction according to the orientation of the target ellipse. This behaviour was observed in the case of visual perturbations although the response in the unperturbed muscle was smaller and delayed relative to the response in the perturbed muscle. The amplitude of the target-dependent reflex component increased over the course of training (paired t-test, df=19, t=1.96, p=0.06), in parallel with improved performance scores (paired t-test, df=19, t=5.22, p<0.001). Neither short nor long latency EMG responses in APB and ADM to peripheral nerve stimulation showed task-specific modulation.

We conclude that optimal control of a myoelectric-controlled interface incorporates both feed-forward (modulation of common drive) and feed-back (minimum intervention controller) strategies. However, when visual feedback is withheld, control is achieved predominantly through feed-forward mechanisms with minimal influence of proprioceptive information.


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**PC92**

**Modulation of the dynamic interaction between STIM1, Orai1 and TRPC1 by the cytoskeleton in HEK-293 cells**

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Store-operated Ca\(^{2+}\) entry (SOCE) is one of the most relevant pathways for Ca\(^{2+}\) influx in non-electrically excitable cells. A conformational coupling mechanism between the endoplasmic reticulum Ca\(^{2+}\) sensor STIM1 and Ca\(^{2+}\) permeable channels in the plasma membrane has been recently reported to account for the activation of SOCE upon depletion of the intracellular Ca\(^{2+}\) stores (Hogan et al., 2010). In addition, TRPC proteins have been shown to associate with Orai1 and STIM1 in a dynamic ternary complex regulated by the occupation of membrane receptors, which might play an important role in the activation of Ca\(^{2+}\) entry (Salido et al., 2011). A number of studies have reported a role for the cytoskeleton modulating the activation of SOCE; therefore, here we have investigated whether the interaction between STIM1 and the Ca\(^{2+}\) permeable channels is modulated by the actin or microtubular network.

Human embryonic kidney 293 (HEK293) cells were obtained from the American Type Culture Collection. Cytosolic free Ca\(^{2+}\) concentration measurement, immunoprecipitation and Western blotting were performed as previously described (Ben-Amor et al., 2006; Woodard et al., 2010).

Cell treatment for 30 min with the microtubular disrupter colchicine (30 μM) significantly enhanced the activation of SOCE by 23% (p<0.05 Student’s t-test; n=6), as well as increased thapsigargin (TG)-evoked association between STIM1 and Orai1 or TRPC1 by 75 and 44%, respectively (p<0.05; n=6). Conversely, stabilization of the microtubules by treatment with 10 μM paclitaxel for 30 min attenuated SOCE by 60% and the interaction between STIM1 and the Ca\(^{2+}\) channels Orai1 and TRPC1 by 50 and 60%, respectively (p>0.05; n=6), altogether suggesting that the microtubules act as a negative regulator of SOCE. Stabilization of the cortical actin filament layer by treatment for 30 min with 10 μM cytochalasin D for 40 min did not significantly modify TG-evoked association between STIM1 and Orai1 or TRPC1 but enhanced TG-stimulated SOCE by 70% (p<0.05; n=6). Finally, inhibition of calmodulin by treatment for 10 min with 1 μM calmidazolium enhances SOCE by 63%. In addition, disruption of the actin cytoskeleton results in inhibition of TG-evoked association of calmodulin with Orai1 and TRPC1. In summary, we demonstrate that the cytoskeleton plays an essential role in the regulation of SOCE through the modulation of the interaction between STIM1 and the Ca\(^{2+}\) permeable channels Orai1 and TRPC1.


Supported by MICINN grant BFU2010-21043-C02-01 and Junta de Extremadura-FEDER CR10010. C.G. and N.D. are supported by Spanish Ministry of Science and Innovation (PTA2010-21043-C02-01) and Junta de Extremadura (PRE09020), respectively.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC93**

**Phosphatidylinositol-4,5-bisphosphate is decreased in insulin resistance provoked by multiple conditions in L6 myotubes and 3T3-L1 adipocytes**

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In insulin resistance, a risk factor for type 2 diabetes, cells fail to respond to insulin. In striated muscle and fat, this manifests as impaired insulin-stimulated glucose uptake due to reduced plasma membrane insertion of the glucose transporter GLUT4. In 3T3-L1 adipocytes and L6 myotubes, levels of the lipid phosphatidylinositol-4,5-bisphosphate (PIP2) are decreased in insulin resistance induced by chronic insulin stimulation1–2. However, the reason for this decrease, and whether other factors that induce insulin resistance affect PIP2, is unknown.

We have investigated whether PIP2 levels are perturbed in insulin resistance induced by several factors (Table 1). The ability of these pre-treatments to abolish insulin-stimulated 3H2-deoxy-glucose uptake was verified, and PIP2 was measured in...
cell extracts by quantitative blotting using a PIP$_2$ probe derived from phospholipase C (PLC) δ$_3$. PIP$_2$ levels were significantly reduced under all conditions tested (Mann-Whitney test). PIP$_2$ is regulated by PIP kinases, which synthesise it, phosphatases, which degrade it, and PLCs, which hydrolyse it. To investigate whether the observed PIP$_2$ reductions resulted from phosphatases, which degrade it, and PLCs, which hydrolyse it, experiments were performed on membrane preparations, using insulin or TNFα to induce insulin resistance in 3T3-L1 cells, and insulin or Angiotensin II in L6 myotubes. For kinase assays, membrane samples were incubated with 32P-ATP and a recombinant PIP-kinase. Lipids were then extracted, separated by thin layer chromatography and quantified by phosphoimaging. Surprisingly, no differences in kinase or phosphatase activity were observed. Thus, although this study shows a correlation between decreased PIP$_2$ levels and insulin resistance induced in 3T3-L1 adipocytes and L6 myotubes by several conditions, the underlying mechanism remains unknown. However, the results are consistent with a possible role for PLC, and experiments to investigate this possibility are ongoing.

Table 1: Conditions used to induce insulin resistance, and the effect on PIP$_2$ levels

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>3T3-L1 PIP$_2$ (% basal; mean±SEM)</th>
<th>L6 PIP$_2$ (% basal; mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3αM, insulin, 24h</td>
<td>55.3±5.1% (P&lt;0.001; n=13)</td>
<td>46.7±0.5% (P&lt;0.005; n=12)</td>
</tr>
<tr>
<td>40ng TNFα, 3h</td>
<td>65.5±5.9% (P&lt;0.001; n=12)</td>
<td>56.1±8.4% (P&lt;0.001; n=7)</td>
</tr>
<tr>
<td>10μM DEX, 1.24h</td>
<td>ND</td>
<td>11.8±2.9% (P&lt;0.001; n=13)</td>
</tr>
<tr>
<td>100μM Angiotensin II, 2.4h</td>
<td>ND</td>
<td>15.7±3.5% (P&lt;0.001; n=13)</td>
</tr>
<tr>
<td>100μM DEX, 3h</td>
<td>56.8±5.2% (P&lt;0.001; n=12)</td>
<td>54.3±4.5% (P&lt;0.001; n=7)</td>
</tr>
</tbody>
</table>


I would like to Diabetes UK for funding, and members of the Hinchliffe lab especially Debbie Grainger and Chris Tavelis. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC94**

**The effect of subnormothermic perfusion on oxidative stress in isolated rat liver**

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Hypothermia can induce reactive oxygen species (ROS) production in cells and tissues (Alva et al.2010). However, the use of deep hypothermia may improve cell protection in clinical situations like ischemia/reperfusion (IR) injury. During the hypothermic IR process, an imbalance in the oxidant/antioxidant levels could lead to oxidative stress and the loss of cell viability and liver function.

We determine the hepatic oxidant/antioxidant balance and liver damage in a model of isolated rat liver after a normothermic (37 °C) and subnormothermic perfusion at 26 °C and 22 °C.

Sprague-Dawley rats were anesthetized with I.P. sodium pentobarbital (50 mg/Kg of body weight) and their livers were removed for perfusion in a non-recirculating system at a flow rate of 4mL/min/g liver. The perfusate used was Krebs-Henseleit buffer (pH 7.4) equilibrated with 95% O$_2$, 5% CO$_2$ (Varietti et al.2006) and the temperature of the medium was adjusted according to the experimental groups: in normothermia group, livers were perfused during 30 min at 37 °C (N37) and in subnormothermia groups for 15 min at 37 °C followed by 15 min at 26 °C (S26) or 22 °C (S22). The perfusate levels of oxidants and the level of damage indicators were measured at the end of each treatment.

As an indicator of liver damage, the alanine aminotransferase (ALT) levels in the perfusate tend to decrease in both subnormothermia (S26 and S22) groups. Nitric oxide values showed a dramatic increase in the perfusate from S22 group and the oxidative damage, measured as malondialdehyde (MDA), was significantly lower in the S22 group when compared to S26 and N37 groups.

Liver damage and oxidative stress decreased with the use of subnormothermic perfusion in an isolated rat liver perfusion system with the best protection found in this study to be 22 °C.

Oxidative stress and damage indicators released into the perfusate at the end of the experiment.

<table>
<thead>
<tr>
<th>Experimental groups:</th>
<th>N37, normothermia</th>
<th>S26, subnormothermia at 26 °C</th>
<th>S22, subnormothermia at 22 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/mL)</td>
<td>1.85±0.025</td>
<td>0.27±0.021</td>
<td>0.17±0.049</td>
</tr>
<tr>
<td>NOx (μmol/mL)</td>
<td>0.48±0.108</td>
<td>0.31±0.027</td>
<td>2.64±0.210**</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>3.69±1.309</td>
<td>4.22±1.703</td>
<td>4.66±2.981</td>
</tr>
</tbody>
</table>

Experimental groups: N37, normothermia; S26, subnormothermia at 26 °C; S22, subnormothermia at 22 °C

Note: Values are mean ± standard error. **P<0.001 versus normothermia

Abbreviations: MDA, malondialdehyde; NOx, nitrate plus nitrite content; ALT, Alanine aminotransferase

Alva et al.(2010) Resuscitation 81, 609-616

Vairetti et al.(2006) J Hepatol 44, 894-901

This work was supported by research grant PI 081389

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**PC95**

**Diabetic modulation of adipose tissues CRP in piglets**

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Inflammation and diabetes are strongly connected with increased level of circulating cytokines. However, still little is known about the interaction between inflammation and adipose tissue. Clinical data showed that plasma levels of the proinflammatory cytokines, such as IL-6 or TNF-α changed with obesity grade and insulin resistance. Previous reports indicated that CRP (C-reactive protein) levels are correlated with body mass index (BMI) and body fat distribution. Additionally, recent studies have shown that elevation of CRP concentrations might be an independent predictive parameter of type 2 diabetes mellitus.

Thus, the present study was undertaken to investigate the effect of hyperglycemia on CRP level in plasma and adipose tissues taken from piglets with induced diabetes.

Studies were performed on the 8 weeks old piglets (Polish Landrace Fatteners, n=18), which were divided into control and two experimental groups. To develop hyperglycemia, animal received ip - injections of streptozotocin (150 mg/piglets over 5 days (75, 0, 50, 0 and 25mg); DM I) or glucocorticoid (60 mg/piglets over 5 days (10, 0, 20, 0 and 30mg); DM II). After completing the experiment (5 days), blood and adipose (vis-
Skeletal muscle carnitine depletion attenuates fat oxidation and increases carbohydrate oxidation in the rat. The results showed that streptozotocin caused the increase of plasma CRP level from 2.51 ± 0.31 ng/ml in control group to 4.03 ± 0.8 ng/ml in DM I (P<0.05). Unexpectedly, the significant decrease in CRP concentrations was observed in visceral adipose tissue (by 58.9%) and epicardial adipose tissue (by 32.5%) (P<0.05). There was significant increase in CRP plasma level in DM II group and similarly to piglets with DM I, significant decrease of CRP concentration was observed in visceral and epicardial adipose tissues, by 77.4% and 56.14%, respectively (P<0.05). The obtained results clearly showed that hyperglycemia modulated the CRP levels in plasma and adipose tissues. The data suggest that adipose tissues might be source of plasma CRP in diabetic piglets and that cytokines potentially play an important role in the mediating metabolic changes associated with insulin resistance and inflammation.

Mildronate impairs carnitine biogenesis and accelerates its renal clearance, reducing plasma and tissue carnitine content. Carnitine, as a principal substrate for carnitine palmitoyl transferase 1 (CPT 1), plays an obligatory role in skeletal muscle long chain fatty oxidation. We hypothesised that pharmacologically lowering skeletal muscle carnitine content would attenuate whole body fatty acid oxidation whilst concomitantly increasing carbohydrate (CHO) oxidation in the rat. Sixteen, 10 week old male lean Zucker rats randomly received either water (CON, n=8) or mildronate supplemented water (MIL, n=8) for 10 days (1.6 g.kg⁻¹ bm⁻¹.d⁻¹ for 2 days and 0.8 g.kg⁻¹ bm⁻¹.d⁻¹ thereafter). On days 7 to 10 animals were housed in Oxymax indirect calorimetry chambers where O2 consumption and CO2 production were measured continuously and substrate oxidation rates calculated thereafter. On day 10, the soleus muscle was excised from both hind limbs under terminal anaesthesia (sodium thiobutabarbital, 125 mg.kg⁻¹ i.p. Inactin™, Sigma) and ‘snap’ frozen in liquid nitrogen. Significant differences between groups were detected using an unpaired Student’s t test.

Compared with CON, soleus muscle carnitine content was 79% lower in the MIL group (4.92 ± 0.12 vs. 1.02 ± 0.05 mmol.kg⁻¹ dm⁻¹, [mean±SEM], P<0.001). During a 12 hour fast on day 10 lipid oxidation was 18% lower in the MIL group compared to CON (8052 ± 128 vs. 6601 ± 122 μmol.kg⁻¹.h⁻¹, P<0.001), while CHO oxidation was increased by 17% (4447 ± 184 vs. 5211 ± 214 μmol.kg⁻¹.h⁻¹, P<0.01). Skeletal muscle glycogen content was 72% lower in MIL compared to CON (30.0 ± 7.4 vs. 8.5 ± 2.9 mmol.kg⁻¹ dm⁻¹, P<0.05).

These novel data demonstrate skeletal muscle carnitine depletion profoundly affects whole body fat and CHO oxidation in vivo in the rat. Carnitine depletion may therefore be a muscle orientated pharmacological approach to increase muscle CHO oxidation in insulin resistant states.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
throcye membranes of T2DM patients compared to healthy controls. Typically, the values (mean ± SEM) were: (A) spectrin-control 1,056 ± 92 and T2DM 3,302 ± 70, (B) ankyrin-control 1,107 ± 69 and T2DM 3,376 ± 40, (C) band 3 protein-control 1,525 ± 56 and T2DM 3,221 ± 28, (D) protein 4.1-control 1,646 ± 128 and T2DM 3,033 ± 23 and (E) glycophrin - control 2,072 ± 124 and T2DM 2,719 ± 20. These results provide evidence of profound quantitative and qualitative alteration of the erythrocyte membrane proteins in T2DM patients compared to healthy controls indicating functional implications of the diabetic patients.


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PC98

Endurance exercise decreases the lipid droplet association with perilipin 2 in human skeletal muscle

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Intramuscular triglyceride-containing lipid droplets (LDs) are an important source of fuel during exercise in human skeletal muscle and a site for lipid storage during periods of elevated lipid availability. LDs exhibit a distinct array of proteins associated with their phospholipid monolayer. Perilipin 2 (traditionally termed ADRP/adipophilin) colocalizes with approximately 60% of LDs in skeletal muscle, while the other LDs do not contain perilipin 2. However, the function of perilipin 2 with regards to the metabolic regulation of skeletal muscle is currently unknown. Therefore, we measured LD and perilipin 2 content in skeletal muscle and examined potential changes in colocalisation in response to moderate endurance exercise. Muscle biopsies were obtained (1% lidocaine local anaesthesia) from 7 lean, healthy males (22 ± 2 years, BMI 24.2 ± 0.9 kg.m-2, VO2 peak 43.8 ± 1.4 ml.min-1.kg-1) before and after 1 h of moderate intensity cycling at ~65% VO2 peak. Five μm cryosections were stained using antibodies targeting perilipin 2, post 0.025 ± 0.004 LDs,μm-2, post 0.027 ± 0.002 LDs,μm-2; P < 0.05), whereas the number of LDs devoid of perilipin 2 detection was unchanged (pre 0.028 ± 0.003 LDs,μm-2, post 0.025 ± 0.004 LDs,μm-2; P = 0.53). In conclusion, this study shows that despite a decrease in skeletal muscle LD content immediately following a single bout of endurance exercise, perilipin 2 content in human skeletal muscle remains unchanged. Furthermore, the colocalisation of perilipin 2 and LDs is reduced post-exercise, apparently due to the preferential lipolysis of perilipin 2 containing LDs. These results are the first to show that perilipin 2 may play a regulatory function in skeletal muscle LD metabolism during exercise.

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PC99

Bruton’s tyrosine kinase participates in the regulation of STIM 1 by tyrosine phosphorylation during SOCE in human platelets

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Stromal interaction molecule 1 (STIM 1) is a key protein for conducting store-operated Ca2+ entry (SOCE) in human platelets that can be phosphorylated on Ser/Thr- and Pro-residues, as recently reported (1,2). In contrast, Tyr-phosphorylation of STIM1 remains poorly described (2), and is the aim of the present study.

Human platelets were obtained from healthy donors, according to the Declaration of Helsinki, as previously described elsewhere (3). Human platelets were stimulated with thapsigargin (TG; 200 nM), and then automatically fixed at different time points using a Quench flow system. Subsequently, STIM 1 immunoprecipitation and Western blotting using a specific anti-phospho-tyrosine antibody (4G10) was performed. Treatment of dimethyl BAPTA-loaded human platelets suspended in a free Ca2+-medium (100 μM of EGTA was added) with TG evoked a maximum STIM1 Tyr-phosphorylation after 2 ± 0.5 s of stimulation. STIM1 located in the plasma membrane presented a similar Tyr-phosphorylation pattern.

Incubation of human platelets for 10 min with LFM-A13 (10 μM), a specific Bruton’s tyrosine kinase (Btk) inhibitor (4), prevented TG-induced STIM1 Tyr-phosphorylation as well as coupling between STIM1 and Orai1, which has been presented as a key step for conducting SOCE in human platelets (n=4-6)(5). Finally, we have used EGS-crosslinker in order to analyze whether Tyr-phosphorylation is required for STIM1 multimerization. Surprisingly, our results indicate that in resting platelets STIM1 is already multimerized. STIM1-multimerization was significantly enhanced by stimulation of human platelets with TG, independently of changes in the cytosolic Ca2+ concentration. Treatment of human platelets with 10 μM LFM-A13 for 10 min was unable to prevent TG-evoked STIM1 multimerization (n=4).

Altogether, our results indicate that Btk-dependent STIM1 Tyr-phosphorylation is required for STIM1 complexing with other elements that participate in SOCE, but not for the STIM1 multimerization.


Nervous system-targeted expression of CGRP/RAMP1 receptors enhances baroreflexes and opposes angiotensin-induced hypertension

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While calcitonin gene-related peptide (CGRP)-induced vasodilation mediated by vascular CGRP receptors is widely recognized, the role of neuronal CGRP receptors in blood pressure (BP) regulation remains unclear. We recently reported that transgenic mice with ubiquitous expression of human receptor-activity-modifying protein 1 (hRAMP1), an obligatory CGRP receptor subunit, exhibit increased baroreflex sensitivity for control of heart rate (BRS-HR) and resistance to angiotensin II (Ang-II) induced hypertension [1]. The contributions of vascular vs. neuronal CGRP receptors to the favorable phenotype could not be definitively determined. The goal of this study was to determine if mice with hRAMP1 selectively targeted to the nervous system by the Nestin promoter [2] also show increased BRS-HR and resistance to hypertension. In addition to measuring BRS-HR (sequence technique), we assessed baroreflex BP buffering capacity – a measurement more relevant to BP control. Radiotelemeters, intravenous catheters and osmotic minipumps were implanted in male hRAMP1 (n=11) and littermate control (n=10) mice under ketamine-xylazine anesthesia. Data were collected before and after 2 weeks of Ang-II infusion via osmotic minipump (1000 ng/kg/min) in conscious mice. Baroreflex BP buffering capacity was calculated as the fold increase in the pressor response to phenylephrine (IV) after vs. before ganglionic blockade (chlorisondamine, 12 μg/g). The depressor response to ganglionic blockade provided an estimate of sympathetic vasomotor tone. Mice were killed with an overdose of pentobarbital at the end of the experiment. Data (mean±SEM) were analyzed using t-tests and repeated measures ANOVA as applicable, with significance taken at P<0.05 (see Table). In control mice, Ang-II infusion increased mean 24-hr BP and sympathetic vasomotor tone, and decreased BRS-HR and baroreflex BP buffering. These deleterious effects were abrogated in hRAMP1 mice. Interestingly, baroreflex BP buffering capacity was markedly enhanced in hRAMP1 mice before as well as during Ang-II. Selective targeting of hRAMP1 to the nervous system was confirmed by RT-PCR and the absence of an enhanced vascular depressor response to CGRP (IV) in hRAMP1 (n=3) vs. control (n=3) mice (data not shown).

In summary: 1) Under basal conditions, baroreflex BP buffering capacity is enhanced in hRAMP1 vs. control mice whereas BRS-HR, sympathetic vasomotor tone and mean BP are normal; and 2) the effects of Ang-II including hypertension, increased sympathetic tone, and impaired baroreflex are abrogated in hRAMP1 mice. The results identify protective autonomic and antihypertensive actions of neuronal CGRP/RAMP1 receptors and encourage targeting these receptors for therapeutic benefit.

<table>
<thead>
<tr>
<th>Ang-II vs baseline, ΔP=0.05</th>
<th>hRAMP1 vs. control, ΔP=0.05</th>
<th>Control</th>
<th>hRAMP1</th>
<th>Control</th>
<th>hRAMP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean 24-hr BP (mmHg)</td>
<td>102±6</td>
<td>102±6</td>
<td>120±6</td>
<td>120±6</td>
<td></td>
</tr>
<tr>
<td>Sympathetic Tonicity (盎格氏)</td>
<td>-41.65</td>
<td>-40.85</td>
<td>-75.4</td>
<td>-75.4</td>
<td></td>
</tr>
<tr>
<td>BRS-HR (second/mg)</td>
<td>7.5±0.3</td>
<td>7.3±0.3</td>
<td>1.7±0.2</td>
<td>1.7±0.2</td>
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</tr>
<tr>
<td>Baroreflex BP buffering</td>
<td>1.28±0.2</td>
<td>1.28±0.2</td>
<td>1.28±0.2</td>
<td>1.28±0.2</td>
<td></td>
</tr>
</tbody>
</table>

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Determinants of human cerebral pressure-flow velocity relationships: new insights from vascular modeling and Ca2+ blockade

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The fundamental determinants of human cerebral pressure-flow relations are not fully understood, particularly the role of vascular mechanical properties in dynamic cerebral autoregulation. Furthermore, whilst the myogenic vascular response is frequently cited as a key determinant of cerebral hemodynamics there are limited experimental descriptions of dynamic pressure-flow relations following cerebral vascular Ca2+ blockade to validate this hypothesis (Zhang et al., 2009). Therefore, we sought to 1) determine whether capacitive blood flow in compliant cerebral vessels driven by the rate of change in blood pressure is an important determinant of middle cerebral artery velocity (MCAv) dynamics, and 2) characterise the impact of vascular myogenic blockade on these cerebral pressure-flow velocity relations in humans. In eight healthy subjects we measured MCAv and mean arterial pressure (MAP) at rest and during oscillatory lower body negative pressure at 0.10 and 0.05 Hz before and after selective cerebral Ca2+ channel blockade (60 mg oral Nimodipine). Cerebral pressure-flow velocity relationships were characterised using transfer function analysis and a regression-based analysis approach based on a two element arterial Windkessel model that incorporates MAP and the rate of change in MAP (dMAP/dt) as predictors of MCAv dynamics. Values are means±SD compared by ANOVA and related using correlation and multiple linear regression analysis. Results show that incorporation of dMAP/dt accounted for a greater proportion of the MCAv variance (R2 range 0.80-0.99) than if only MAP was considered (R2 range 0.05-0.90). Ranking of the standardized beta-coefficients showed that the compliance term was always ranked higher than the conductance term under both control and Ca2+ blockade conditions. Transfer function coherence under the control condition was >0.78 during both OLBNP frequencies and the transfer function phase lead of MCAvmean on MAP was seen in all subjects. The capacitive gain relating dMAP/dt and MCAv was strongly correlated to transfer function gain (0.05 Hz, r=0.93, p<0.01; 0.10 Hz, r=0.91, p<0.01) but not to the phase or coherence. Ca2+ blockade increased the conductive gain relation between MAP and MCAv (-0.00080±0.21 vs. 0.20±0.35, p<0.05) and reduced transfer function phase at 0.05Hz (1.3±0.62 vs. 0.90±0.50 radians, p<0.01). However, capacitive gain and transfer function gain were unaltered. These findings indicate that volume change in compliant cerebral vessels may be an important determinant of dynamic cerebral pressure-flow relations. Data also indicate that Ca2+ channel blockade enhances pressure-driven resistive blood flow that may render the cerebral microcirculation more vulnerable to systemic blood pressure fluctuations.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Poster Communications

PC102

Time course of vascular reactivity in hypertensive rats induced by ethanol treatment

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Several studies have shown that chronic ethanol intake is associated with cardiovascular dysfunctions, autonomic changes and hypertension. However, the mechanisms involved in ethanol intake-related blood pressure increase are not yet completely understood and can be caused by a myogenic mechanism, which involves alteration of contractile properties of vascular smooth muscle. This study evaluated basal arterial blood pressure and heart rate, as well as cardiovascular responses to infusion of vasoactive agents in unanesthetized rats maintained under chronic ethanol treatment. Male Wistar rats (250g) were treated with ethanol for 4 weeks: first week 5%, second week 10% and the last two weeks 20% of ethanol. One day before the experiments the rats were anesthetized with tribromoethanol (250 mg/kg) and a catheter (4-cm segment of PE-10 heat-bound to a 13-cm segment of PE-50, Clay Adams) was inserted into the abdominal aorta through the femoral artery, for blood pressure and heart rate recording. Whenever intravenous administration of drugs was necessary, a second catheter was implanted into the femoral vein. Both catheters were tunneled under the skin and exteriorized at the animal’s dorsum. On the day of the experiment the animals were injected with phenylephrine (50 μg/kg/mL) or sodium nitroprusside (70 μg/kg/mL) using an infusion pump. During the treatment the animals had a progressive increase of basal values of mean arterial pressure (MAP) since one week of treatment without alteration of heart rate in basal conditions. The pressor effect of intravenous phenylephrine (Emax) was enhanced in ethanol-treated rats at all different times of treatment, when compared to respective control group. The hypertensive response to intravenous sodium nitroprusside (Emax) was enhanced in ethanol-treated rats only after the fourth week of treatment when compared to respective control group. In summary, these results indicate that ethanol chronic treatment promotes a mild hypertension that could be seen as soon as after the first week of treatment. This increase in MAP was due to the increase in both systolic and diastolic pressures. The vascular responsiveness to the pressor agent phenylephrine was enhanced since the first week of ethanol treatment, demonstrating that the system is very sensitive to pressor agents. Changes in relaxation responses are likely to be an adaptive mechanism to refrain the progressive
increase of blood pressure, contributing to the mild hypertension induced by long-term exposure to ethanol. This change in relaxation could be also a response for the deficit in the nitric oxide endogenous system. These results indicate that the cardiovascular system counteracts the effects induced by chronic ethanol consumption altering mechanisms involved in the maintenance of the vascular tonus. In our study hypertension is an early event of chronic ethanol consumption.


Technical Assistance: Maria Valci Aparecida, Milene Mantovani.

Financial Support: FAPESP and CNPq.

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**PC104**

Curcumin decreased vascular responses to sympathetic nerve stimulation in the mesenteric vascular bed of normotensive and hypertensive rats

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Curcumin is a major active compound derived from the spice turmeric. Previous studies have shown that curcumin exhibits a cardiovascular protective effect, however, little is known with regard to its effect on a vascular dysfunction in a hypertensive condition. We hypothesized that curcumin modulates vascular responses to sympathetic nerve stimulation in normotensive and hypertensive rats. Male Sprague-Dawley rats (220-225 g) were induced hypertension by administering L-NAME (50 mg/kg/day, 3 weeks) in drinking water while normotensive rats were given distilled water. Rats with systolic blood pressure (SBP) higher than 225 ± 2 mmHg (tail cuff measurement) were considered to be hypertensive. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) followed by exsanguinations. Mesenteric vascular beds from both normotensive and hypertensive rats were isolated and set up for perfusion (1). After chemical removal of vascular endothelium by sodium deoxycholate (1.8 mg/ml, 30s), preparations were pretreated with capsaicin (10-7 M) to deplete sensory neurotransmitters and to desensitize vanilloid receptors (2). Contractile responses to electrical field stimulation (EFS 5-40 Hz, 90V, 1 ms for 30s, at 5-min intervals) were performed. A second frequency response curve was generated after a further 30 minutes and served as a control. Effects of curcumin (10-6 M) on sympathetic nerve mediated vasocostriction were tested. Dose-dependent contractile responses to exogenous noradrenaline (NA) (1 μmol-1 mmol) or phenylephrine (PE) (1 μmol-1 mmol) were examined. L-NAME induced-hypertensive rats showed higher SBP compared to normotensive rats (178±5 vs. 121±2 mmHg, p<0.001, n=21, unpaired t-test). Perfusion pressure of hypertensive preparations was significantly higher than that of normotensive preparations (37±2 vs. 26±2 mmHg, p<0.05, n=21, unpaired t-test). Curcumin significantly attenuated sympathetic nerve mediated-responses (p<0.05, n=6, ANOVA) and contractile responses to exogenous NA and PE in all preparations (p<0.05, n=6, ANOVA). We have shown that curcumin exhibits an inhibitory effect on sympathetic neurogenic vasoconstrictor responses in normotensive and hypertensive rat mesenteric vascular beds and it may not have direct effect on the availability of NA. This inhibitory effect is likely to involve the postjunctional site inhibition. Thus, the direct effect of curcumin on vascular responses and the underlying mechanisms need to be evaluated in the rat perfused mesenteric vascular bed under raised tone conditions.


This work was supported by a grant from Invitation Research Fund, Faculty of Medicine, Khon Kaen University, Thailand

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**PC105**

An AC8/Orai1 signalling complex underlies dynamic Ca2+-regulated cAMP signalling

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Ca2+ entry attributed to the activation of store-operated Ca2+ (SOC) channels within the plasma membrane, plays a critical role in numerous cellular functions. Orai1, a 33 kDa protein, is the pore-forming subunit of SOC channels (1,2). An important consequence of SOC channel mediated entry (SOCE) is the regulation of Ca2+-sensitive adenyl cyclases (ACs). These ACs are responsive to sub-μM Ca2+ concentrations and are uniquely selective for SOCE over other modes of Ca2+ rise (3). Recent data from our laboratory using AC-targeted Ca2+ sensors has provided evidence of an intimate association between the Ca2+-stimulated AC8 and SOC channels in discrete ‘Ca2+ microdomains’ (4). The aim of our present study was to examine if there is a direct interaction between AC8 and Orai1 that underpins the functional dependence of AC8 on SOCE. All experiments were performed in cultured HEK293 cells.

Using a multi-disciplinary approach of GST pull-downs, coimmunoprecipitation, FRET and peptide array analysis we demonstrate a direct binding between the N-termini of AC8 and Orai1 that underpins the functional dependence of AC8 on SOCE. All experiments were performed in cultured HEK293 cells.

Live-cell imaging with AC8-targeted biosensors for Ca2+ (GCaMP2-AC8 (4)) or cAMP (Epa2-camps-AC8 (5)) was performed using a wide-field EM-CCD camera-based imaging system. Measurements obtained using GCaMP2-AC8 illustrated a rapid detection of SOCE with a time to peak of 16.4 ± 2.0 s (n=80 cells). In contrast, GCaMP2-AC8 detected little Ca2+
increase during 300μM CCh-evoked ER Ca2+ mobilization in Ca2+-free conditions. When GCaMP2 was tethered to an N-terminally truncated form of AC8 (GCaMP2-8M1, n=120 cells) the time to peak SOCE increased to 43.3 ± 1.8 s (p<0.0001, compared to GCaMP2-AC8). Ca2+ changes seen within the AC8-microdomain during SOCE were paralleled by enhanced cAMP production, detected by an AC8-targeted FRET-based cAMP biosensor. Epa2-camps-AC8. Knockdown of Orai1 expression attenuated the SOCE detected by GCaMP2-AC8 by 76 ± 4% (p<0.0001 compared to control) and AC8-mediated cAMP production by 61 ± 5% (p<0.001 compared to control). SOCE measured globally was reduced by > 90%, suggesting that any remaining Orai1 protein preferentially associates with AC8. We conclude that the longstanding functional dependence of AC8 on SOCE originates from a direct protein-protein interaction between AC8 and Orai1. Such intimacy ensures dynamic, coordinated changes in levels of Ca2+ and cAMP in cells expressing Ca2+-regulated ACs. This study provides the first demonstration that Orai1 can function as an integral component of larger signalling complexes and has major implications for how Ca2+-dependent signalling events may be organized.


This work was funded by the Wellcome Trust

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PC106

Characterising nucleotide release from the EA.hy926 human endothelial cell line during metabolic stress

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During hypoxia and cellular damage, ATP is released into the extracellular environment (1, 2). Once released, ATP acts as a signalling molecule via P2 purinoceptors. The aim of this study is to confirm ATP release under cellular stress and to investigate the presence of other nucleotides, such as ADP. Nucleotide release was measured from confluent cells grown in multi-well plates after incubation in a Na+ HEPES-buffered physiological saline for 60 minutes. During the incubation period, the cells were poisoned with a cocktail of sodium cyanide (4mM), 2-deoxy-D-glucose (10mM) and ionomycin (5μM) for 5-40 minutes.

A sample of the buffer was added to ATP monitoring reagent (AMR, ViaLight®). The light emitted (RLU) was measured using a Berthold tube luminometer (LB955). ADP was measured after conversion to ATP, using phosphoenolpyruvate and pyruvate kinase. The RLU was re-measured and the corresponding ATP RLU subtracted. Calibration curves for ATP and ADP were constructed and used to convert the RLU to nucleotide concentrations. Released nucleotides are subject to interconversion enzymes including, alkaline phosphatase (AP) and ATPases (which dephosphorylate ATP) or ecto-adenosine kinase, nucleoside diphosphokinase and F1F0 ATP synthase (which generates ATP from ADP) (3, 4). ATP and ADP assays were therefore carried out in the presence and absence of the ATP hydrolysis inhibitors, levamisole (10mM), ebsebun (30μM) and ARL 67156 (100μM). ATP and ADP concentrations per 106 cells are presented as means ± S.E.M. The data shows that after 40 minutes, metabolic poisoning elevates the observed extracellular concentration of ATP from 6.5±2nM to 87±12nM and ADP from 893±178nM to 5244±618nM. ATP hydrolysis inhibitors further significantly elevated both extracellular nucleotides. Levamisole increased ATP to 159±21nM and ADP to 7176±893nM. Ebsebun increased ATP to 190±6nM and ADP to 7714±330nM. ARL 67156 increased ATP to 165±12nM and ADP to 7858±189nM (p<0.01 for each when compared with the nil control, ANOVA, n=6).

Since both ATP and ADP are elevated in the presence of the ATP hydrolysis inhibitors, it is not yet clear whether the extracellular ADP is derived from extracellular hydrolysis of ATP or whether it is ‘released’ directly from the cells.

Rogers J. Thompson, et al. (2006) Science 312, 924-27

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PC107

Sulforaphane modulates redox signalling in human aortic adventitial fibroblasts

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Migration and proliferation of adventitial fibroblasts (AF) contributes to vascular remodelling [1] which may be mediated by enhanced generation of reactive oxygen species (ROS) [2]. Sulforaphane (SFN), an isothiocyanate present in cruciferous vegetables such as broccoli, protects cells against oxidative stress and inflammation through activation of the transcription factor Nrf2 [3] which mediates induction of antioxidant defence pathways such as glutathione (GSH) and heme oxygenase-1 (HO-1) via activation of antioxidant response elements [4]. GSH is the predominant intracellular antioxidant and HO-1 catalyses the pro-oxidant heme to generate the vasodilator carbon monoxide and antioxidant biliverdin. To address whether SFN can elicit changes in redox signalling in primary cultured human AF, cells were treated (0-24 h) in medium containing SFN (0-10 μM). Cellular HO-1 and nuclear Nrf2 protein expression were determined by western blot analysis while total GSH levels were measured using a fluorometric assay. Treatment of AF with SFN (2 h) elicited a marked 2 fold increase in nuclear Nrf2 levels with a concomitant significant decrease (50%, 2-8 h) in GSH levels which returned to baseline by 24 h, while HO-1 expression was significantly increased by 2.4 fold in AF treated with 5-10 μM SFN for 8-24 h (n=3, p<0.05, Student’s paired t-test). These findings demonstrate for the first time that SFN can elicit changes in redox signalling in human aortic AF involving Nrf2 activation. Dietary SFN may therefore contribute to protection against...


Mann et al. (2009). Curr Opin Pharmacol. 9, 139-145.

This study is supported by the British Heart Foundation

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**PC108**

**Immunophilin proteins participate in platelet aggregation by regulating granule secretion and calcium homeostasis**


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The immunophilin family has been targeted by using macrolide lactone derivates, such as cyclosporin A (CsA), tacrolimus (FK506) and everolimus (Rapamycin), in order to prevent organ rejection in transplanted patients (1,2). FK506 and CsA have been presented as efficient immunosupressor by altering lymphocyte T function, since both immunophilin blockers prevent calcineurin (CNa) activation (3). Here we analyze in in vitro assays whether inhibition of immunophilins would deregulate cytosolic free calcium concentration ([Ca$^{2+}$]$_{i}$) homeostasis, granules secretion and, subsequently, impair human platelets aggregability.

Human platelets were isolated from healthy donors following the guidelines of Helsinki’s Declaration, as previously described elsewhere (4) and then were incubated with 2 μM of fura-2 AM for 45 min at 37°C or left untreated. Changes in [Ca$^{2+}$]$_{i}$ were monitored using a spectrofluorophotometer exciting the cells alternatively at 340/380 nm and emission was detected at 510 nm. Alpha- and dense-granules secretion was determined by flow cytometry (FACScan cytometer); meanwhile platelet aggregation experiments were performed in a Chrono-log aggregometer.

Human platelets were incubated for 5 min with either the vehicle or FK506 (50 μM). FK506 increased Ca$^{2+}$ release evoked by thrombin (Thr, 0.1 U/ml) by a 60 ± 22.4% (P<0.001 Student’s t-test, n=4-6) compared to controlFK506-untreated platelets. However, immunophilin antagonists reduced Thr-evoked Ca$^{2+}$ entry by 67.8 ± 11.5% (P<0.001, n=6).

Stimulation of human platelets with different Thr concentrations (0.001-0.5 U/ml) reported a different degranulation pattern of alpha- and dense-granules, which were monitored by flow cytometry using PE-labeled anti-P-selectin antibody (anti-CD62) and mepacrine, respectively. Additionally, incubation of platelets with 50 μM of FK506 for 5 min significantly reduced Thr (0.1 U/ml) evoked both alpha- and dense-granule secretion.

Finally, we have found a reduction in Thr-evoked aggregation in the presence of FK506, compared with control untreated cells but stimulated with Thr (0.01 U/ml), perhaps as consequence of the previous inhibitory effects of FK506 on granule secretion.

In summary, we provide evidences for an important role of immunophilins in platelet secretion and aggregation. Dandel M et al (2010), Transpl Immunol. 23:93-103.


Supported by MEC (BFU2010-21043-C02-01) and Junta de Extremadura-FEDER (GR10010 and PRI-B10200). Redondo PC and Lopez E are supported by MEC (RYC2007-00349) and ISCIII (F110/00573), respectively. Berna-Erro A is supported by University of Extremadura Posdoc-research contract (D-01)

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**PC109**

**Modulation of HEK293 proliferation by Kv1.3 channels expression**


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The plasticity of vascular smooth muscle cells (VSMCs), that can switch from a contractile to a proliferative phenotype, associates with several cardiovascular diseases. This phenotypic switch involves changes in the expression of membrane receptors and ion channels. We have previously reported that in mouse femoral VSMCs, upregulation of Kv1.3 channels associates with increased proliferation. Furthermore, this pro-proliferative effect of Kv1.3 may be a conserved phenomenon among vascular beds from different species, as Kv1.3 blockade with specific drugs has antiproliferative effects.

To explore the mechanism linking Kv1.3 expression to cell proliferation, we examined whether heterologous overexpression of Kv1.3 channels also modulates proliferation of human embryonic kidney cells (HEK293). The complete coding sequence of Kv1.3 was subcloned in different vectors, to obtain Kv1.3 expression associated to reporter genes (dsRed-IRES-Kv1.3) or Kv1.3 fusion proteins (pEGFP-N3-Kv1.3 and Kv1.3-N3-myc). Confocal microscopy images of transfected cells demonstrated the plasma membrane location of the fusion proteins. The effects of Kv1.3 on proliferation were characterized by means EdU incorporation and cell-counting assays in mock-transfected, control cells (transfected with the empty vector) and Kv1.3-transfected cells. Kv1.3 overexpression lead to a significant increase in HEK293 proliferation between 24 and 48 hours after transfection, as a consequence of a decrease of the duplication time of the cell cultures (from 15 hours in control to 10 hours in Kv1.3 expressing cells). Moreover, the effect was inhibited by specific blockade of Kv1.3.

Since our previous studies in native VSMCs showed no correlation between changes in membrane potential and cell proliferation, we generated mutant constructs of Kv1.3 in which either permeation (pEGFP-N3-Kv1.3-W389F) or gating and permeation (pEGFP-N3-Kv1.3-AYA) were removed. Patch-clamp studies showed that both constructs expressed non-conducting Kv1.3 channels. In addition, Kv1.3-AYA failed to produce detectable gating currents, while Kv1.3-W389F showed gat-
Reduced P2X receptor-mediated responses in renal vascular myocytes of spontaneously hypertensive rats

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P2X receptors (P2XRs) mediate sympathetic control and autoregulation of the renal circulation triggering contraction of renal vascular smooth muscle cells (RVSMCs) by elevation of [Ca2+]i [1]. We have recently demonstrated that elevation of [Ca2+]i, following activation of ionotropic P2XRs in RVSMCs is caused by Ca2+ entry through P2XRs and voltage-gated Ca2+ channels (VGCCs) and Ca2+-induced Ca2+ release from the sarcoplasmic reticulum (SR) mediated not only by ryanodine receptors (RyRs), but also by inositol 1,4,5-trisphosphate receptors (IP3Rs) [2]. The aim of this work was to compare responses induced by stimulation of P2XRs with 10 μM of αβ-meATP in RVSMCs freshly isolated from spontaneously hypertensive rats (SHR) and their normotensive control, the Wistar Kyoto (WKY) rats. RVSMCs were isolated from arcuate and interlobular arteries of rat kidney, as previously described [3]. P2X-mediating cationic current (I_{P2X}) was recorded using amphotericin B perforated patch-clamp method. Changes of [Ca2+]i in fluo-3 loaded RVSMCs were visualised using fast (33-40 Hz) x-y confocal imaging. Data are presented as mean ± S.E.M. The data groups were compared using unpaired Student’s t-test. We found significant (p<0.01) reduction of I_{P2X} in RVSMCs from SHR: the peak current density was on average 57±7 pA/pF (n=18) in RVSMCs from SHR and 101±12 pA/pF (n=25) in RVSMCs from WKY. The peak amplitude of the αβ-meATP-induced [Ca2+]i transients was also significantly (p<0.001) reduced in SHR RVSMCs: mean ΔF/F0 was 1.6±0.1 (n=89) in RVSMCs from SHR and 3.9±0.2 (n=95) in RVSMCs from WKY. Relative contribution of Ca2+ entry through P2XRs to the αβ-meATP-induced elevation of [Ca2+]i was decreased in RVSMCs from SHR, while contribution of Ca2+ entry via VGCCs was increased. In spite of decreased SR Ca2+ load (tested with 10 mM caffeine) in RVSMCs of SHR, relative contribution of the SR Ca2+ release to the αβ-meATP-induced [Ca2+]i mobilisation was similar in both groups. Nevertheless, 100 mM ryanodine reduced the αβ-meATP-induced [Ca2+]i transients significantly stronger in SHR RVSMCs, while the effect of 30 mM 2-APB was similar in both groups. Our results suggest that in SHR: (1) both Ca2+ entry and Ca2+ release following P2X receptor activation in RVSMCs were significantly reduced and (2) the main cause of the Ca2+ release reduction was decrease of the SR Ca2+ load resulting from an enhanced RyR-mediated Ca2+ leak. Reduction of P2X2-mediated signals may underlie impairment of sympathetically driven and autoregulatory responses in renal vasculature leading to glomerular damage in hypertension.


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Induction of vascular antioxidant defences in response to dietary lipids and atherogenesis in hypercholesterolemic rabbits

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Atherosclerosis and complications such as stroke and myocardial infarction are major causes of the death worldwide. Besides several genetic and environmental factors, increased serum cholesterol and oxLDL are considered to be inducing factors of atherosclerosis (1). We have previously reported a significant increase in CD36 mRNA levels in cholesterol fed rabbits and shown that vitamin E pretreatment prevented the dietary lipid induced increase in CD36 mRNA expression. In the present study, we further investigated redox signalling pathways involved in cellular defence against oxidative stress associated with atherogenesis in hypercholesterolemic rabbits. All experimental procedures were approved by the Marmara University Ethics Committee. Twenty-one male albino rabbits were assigned randomly to three groups fed: (i) vitamin E deficient diet, (ii) vitamin E deficient diet containing 2% cholesterol, and (iii) vitamin E deficient diet containing 2% cholesterol with daily intramuscular injections of vitamin E (50 mg/kg). After four weeks, serum cholesterol and vitamin E levels were determined. Supplementation with cholesterol resulted in ~30-fold increase of plasma cholesterol while vitamin E treatment increased serum vitamin E levels 11-fold (mean±S.D., n=7, p<0.001, Student’s t-test). When thoracic aortae stained with hemotoxylene eosin were examined by light microscopy, cholesterol fed rabbits exhibited atherosclerotic lesions and endothelial damage compared to control rabbits. Notably, lipid accumulation and foam cell formation was detectable in animals fed cholesterol and treated with vitamin E. The consequences of hypercholesterolemic diet were further examined determining protein levels of Nr2f2 and MMP-1 (n=3 per group) by immunoblotting and PPARγ, ABCA1 and MMP-1 mRNA levels (n=5 per group) by quantitative RT-PCR. Nr2f protein expression was increased in the cholesterol group. Both MMP-1 protein and mRNA expression was
increased in cholesterol group but decreased in cholesterol and vitamin E treated group. PPARγ and ABCA1 mRNA levels were decreased in the cholesterol group and increased in vitamin E treated group. Enhanced expression of Nr2f2 supports our previous findings of Nr2f regulated CD36 expression (2) and may reflect the fact that Nr2f may be proatherogenic (3). Increased protein and mRNA expression of MMP-1 in cholesterol fed group may underlie its key role in extracellular matrix remodelling in atherosclerosis (4). As PPARγ is known to inhibit MMP-1 via the AP-1 pathway (5), this may account for our finding of decreased PPARγ in hypercholesterolemic rabbits. Our findings suggest that vitamin E may afford protection in part by decreasing MMP-1 expression and increasing PPARγ and ABCA1 expression.


Supported by TUBITAK COST B 35-5(106S121)

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PC112

β-NAD mediates purinergic post-junctional enteric inhibitory responses via P2Y1 receptors in the colon

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Inhibitory motor neurons regulate complex motor patterns in the GI tract such as peristalsis, receptive relaxation, and sphincter opening. The neurotransmitters responsible for inhibitory responses have been investigated for decades, and numerous substances proposed. β-nicotinamide adenine dinucleotide (β-NAD) has recently been suggested to mediate inhibitory neurotransmission in mouse and human colons via P2Y1 receptors (P2Y1R; Hwang et al., 2011). However, evidence supporting a role for P2Y1 receptors in enteric responses is based on pharmacological agents, MRS2179 and MRS2500, thought to be highly specific P2Y1R antagonists. In order to confirm (i) the role of P2Y1R in inhibitory responses, and (ii) the importance of P2Y1R in colonic transit, we compared post-junctional responses of wildtype (WT) and P2Y1R−/− mice using intracellular and isotropic force recordings in vitro. Video imaging with spatio-temporal analysis (STMap) was used to examine how loss of P2Y1R affects colonic transit. Electrical field stimulation (EFS; 0.1-0.5 m:s;150V) of enteric inhibitory nerves evoked bi-phasic inhibitory junction potentials (IJPs) in mouse and cynomolgus monkey colons. IJPs consisted of an initial fast hyperpolarization, followed by a slower secondary hyperpolarization. Both phases of the IJP were blocked by tetrodotoxin (TTX; 1 μM), and the secondary hyperpolarization blocked by N(o)-nitro-L-arginine (L-NAME;100 μM), whereas the initial IJP was inhibited by MRS2500 (1 μM). In colons of P2Y1R−/− mice EFS produced large atropine (AT, 1 μM) sensitive excitatory junction potentials (EJPs), followed by slow, L-NAME sensitive IJPs. In AT and L-NAME, post-junctional responses were abolished in P2Y1R−/− mice. Force measurements revealed abnormal activity in colons of P2Y1R−/− mice. EFS (5 Hz; 0.3 ms, 30 s) of P2Y1R−/− colons caused relaxation that was potentiated by AT and inhibited by L-NAME. In AT and L-NAME, relaxation was abolished in P2Y1R−/− mice compared to the relaxations of WT muscles. Picospritzing ATP or ADP onto WT or P2Y1R−/− muscles caused hyperpolarization that was not affected by TTX or MRS2500, however picospritzing β-NAD caused hyperpolarization in WT muscles, but these agents did not cause hyperpolarization in P2Y1R−/− muscles. Video imaging and STMaps revealed fecal transit of 0.53±0.06 mm s−1. Transit was disrupted in colons of P2Y1R−/− mice; pellets traveled to a point mid way along the colons at 0.25±0.07 mm s−1 (P<0.05, t-test), where transit terminated. These data reveal the importance of P2Y1R in colonic motor responses. P2Y1R mediate the fast component of IJPs. Responses mediated by P2Y1R were mimicked by β-NAD, but not to ATP or ADP. P2Y1 receptors represent an important transduction mechanism for enteric inhibitory responses and are critical for normal colonic transit.


Supported NIH DK41315.

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PC113

Calcium mobilization by NAADP from acidic stores in the megakaryoblastic cell line MEG01

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Changes in cytosolic free Ca2+ concentration ([Ca2+]i) are among the most universal signaling events regulating cell function. Activation of membrane receptors increases [Ca2+]i by Ca2+ release from agonist-sensitive Ca2+ compartments and Ca2+ entry through plasma membrane channels. Ca2+ release from the endoplasmic reticulum (ER) occurs through the occupation of two types of Ca2+-release channels, namely inositol 1,4,5-trisphosphate (IP3) and ryanodine receptors (RyR), by IP3 and cyclic ADP-ribose, respectively (Cancela, 2001). A novel family of intracellular Ca2+-release channels termed two-pore channels (TPCs) have been presented as the receptors of nitoacin acid adenine dinucleotide phosphate (NAADP), the most potent Ca2+ mobilizing intracellular messenger (Churchill et al., 2002). TPCs are exclusively localized to the endosomial system mediating NAADP-evoked Ca2+ release from the acidic compartments. We have investigated NAADP-mediated Ca2+ release from intracellular stores in the megakaryoblastic cell line MEG01.

Changes in cytosolic and intraluminal free Ca2+ concentrations were registered by fluorometry using fura-2 and rufa-fluorescence, respectively, and TPC expression was detected by reverse transcription polymerase chain reaction (RT-PCR). Treatment of MEG01 cells with the H+/K+ ionophore nigericin or the V-type H+−ATPase selective inhibitor bafilomycin A1 results in a rise in cytosolic free Ca2+ concentration (n=6), which revealed the presence of acidic Ca2+ stores in these cells. The acidic stores were also sensitive to the SERCA inhibitor 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ). NAADP releases Ca2+ from TBHQ-sensitive acidic stores in MEG01 cells probably mediated by the activation of TPC1 and TPC2, whose expression was detected by RT-PCR. Ca2+ efflux from the TBHQ-sensitive acidic Ca2+ stores or the TBHQ-insensitive ER results in ryanodine-
Homer1 has a role in Store Operated Calcium Entry in human platelets

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Store-operated calcium entry (SOCE) is a process controlled by the filling state of intracellular calcium stores and mediated by a complex of signalling proteins, including the endoplasmic reticulum calcium sensor STIM1 and the calcium permeable channels Orai1 and TRPCs (Salido et al., 2011). Homer1 is a scaffolding protein that participates in targeting and localisation of ryanoide receptors, IP3 receptors, STIM1 and TRPs via its N-terminal EVH1 domain that interacts with the proline-rich sequences of the PPXXF motif which are found in many target and ligand proteins (Worley et al., 2007; Gasperini et al., 2009; Shalygin et al., 2010). Here we have investigated the role of Homer1 in SOCE and its association and subsequent modulation of calcium signal proteins in human platelets. Platelets were obtained from blood drawn from healthy drug-free volunteers with approval of the local ethical committees and in accordance with the Declaration of Helsinki. Intracellular free calcium concentration ([Ca2+]c) was determined by spectrofluorimetry and protein-protein interactions by Western blotting. In a Ca2+-free medium thapsigargin evoked a prolonged increase in [Ca2+]c and increased the association between Homer1 and IP3R, hTRPC1, STIM1 and Orai1. The subsequent addition of Ca2+ (300 μM) to the external medium induced a sustained increase in [Ca2+]c indicative of SOCE. Electrotransjection of PPKKFR, a specific peptide that emulates the proline-rich sequences of the PPXXF motif that interacts with Homer1 impairing its interaction with other proteins, resulted in an attenuation of thapsigargin-stimulated interaction between STIM1 and Orai1 by 20% and significantly reduced SOCE by 34% (p<0.05 Student’s t-test; n=4). This effect was not found when we used the negative control peptide PPKKRR. Summarizing, Homer1 acts as a scaffolding protein for the SOCE signalling complex in human platelets.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Species variations in TRPC4 properties

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Transient receptor potential (TRP) channels are a large superfamily of nonselective cation channels, most of which are permeable to calcium. They form ubiquitous receptor- and store-operated channels, as well as more specialised receptors in some cell types (e.g. neuronal thermosensors) (Venkatachalam & Montell, 2007). TRPC4 and related TRPC5 are receptor-operated channels mediating both membrane depolarisation and intracellular Ca2+ increases involved in the regulation of vascular tone and endothelial permeability, neurotransmitter release, and gastrointestinal smooth muscle cholinergic excitation. We have previously investigated mouse TRPC4 isoforms, the full-length mTRPC4α and shorter mTRPC4β, and noted differences in their regulation by PIP2 (Otsuguro et al., 2008). We have now cloned three TRPC4 isoforms from the guinea-pig designated cpTRPC4α, β and γ. Mouse and guinea-pig TRPC4 channels are 95% identical, while most differences are localised to their C-termini. Investigation of these natural differences may provide new insights into TRPC4 structure-function relations and their species variations.

The above described TRPC4 isoforms were stably expressed in HEK293 cells grown under culture conditions and recorded using symmetrical Cs+ solutions (125 mM) with intracellular Ca2+ “clamped” at 100 nM with 10 mM BAPTA (Otsuguro et al., 2008). Channels were activated by infusion of GTPγS (0.2 mM) via patch pipette. All TRPC4 isoforms formed functional channels, although current densities at maximal activation (HP=40 mV) varied from about 30 pA/pf (mTRPC4β) to 80 pA/pf (mTRPC4α and cpTRPC4γ). Voltage-dependent properties were investigated in detail by measuring steady-state I-V relationships (6 s ramps from +80 to -120 mV), converting them into the conductance curves which can be approximated by the Boltzmann relation. Although the voltage dependence was identical in all isoforms (slope of 15-19 mV), the potential of half-maximal activation (V1/2) varied between mouse and guinea pig isoforms. Most interestingly, activation of mTRPC4 did not involve a change in V1/2, while cpTRPC4γ showed a negative shift of ~25 mV of its V1/2 during increased G protein activity. At the same time, the initial V1/2 value (i.e. at low G protein activation) was more negative in mTRPC4α (-65.3±2.1 mV, n=10) compared to cpTRPC4γ (47.9±6.8 mV, n=8) and mTRPC4β (-47.3±5.3 mV, n=6) (P<0.02). These differences prompted us to create and investigate two chimeric variants: the cpTRPC4 with the mouse C-terminus chimerically mimicked most closely the V1/2 behaviour seen in wild-type mTRPC4, while the mTRPC4 with the guinea pig C-terminus chimera mimicked that of cpTRPC4γ.

The results suggest that the Cterminus of TRPC4 mainly determines the position of the activation curve on the voltage axis and, likely, its regulation via G protein activation.


Supported by NIH grant R01 DK081654

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Role of Kv1.3 channels in the proliferation of human vascular smooth muscle cells

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Vascular smooth-muscle cells (VSMCs) contribute significantly to occlusive vascular diseases by virtue of its ability to switch from a contractile phenotype to a cell that migrates and proliferates narrowing the vessel lumen. An important element of this phenotypic modulation is a switch in ion transport systems.

Our previous work has explored this aspect in murine VSMCs from femoral arteries. We identified two genes, Kv1.3 and Kv1.2, that were up-regulated in proliferation. Functional studies demonstrated that the increased Kv1.3 currents in these cells were an essential component of their migratory and proliferative phenotype.

In this study, we explored if the association between the phenotypic switch and the upregulation of Kv1.3 channels is conserved in human VSMCs from different vascular beds. We had access to uterine, coronary and renal arteries and saphenous veins from donors, through the Human Collection of Vascular Samples (COLMAH-HERACLES). All the protocols were approved by the Human Investigation Ethics Committees of the participant Hospitals, and conform to the principles outlined in the Declaration of Helsinki. The pieces were cleaned of endothelial and connective tissues and used directly or explanted to obtain cultured VSMCs.

The expression levels of Kv1.3 and Kv1.2 mRNA was determined by real-time qPCR with Taqman® probes in both cultured and contractile VSMCs from the different samples. Protein expression levels were analyzed by western blot with specific antibodies. In addition, the functional expression of Kv1.3 channels was explored with patch-clamp techniques, quantifying the effect of selective Kv1.3 blockers (PAP-1 and Margatoxin) on the total outward K+ currents. Finally, the contribution of Kv1.3 channels to VSMCs proliferation was determined by exploring the effect of these selective blockers on the rate of BrdU incorporation to VSMCs in culture.

Our results show that Kv1.3 and Kv1.2 contribute to the phenotypic switch of VSMCs in all the vascular beds studied. We observed that Kv1.3 mRNA becomes the predominant Kv1 message expressed in proliferating VSMCs, a switch that parallels the changes in Kv1.3 protein expression. Moreover, in all the vascular beds the selective blockade of Kv1.3 channel decreases the rate of cell proliferation without causing a significant increase in apoptotic cell death. We conclude that the functional expression of Kv1.3 channels in proliferating VSMCs is a common feature of different human vascular beds, so that new therapeutical approaches using selective blockers of Kv1.3 channels could represent an advantageous strategy to prevent unwanted remodeling.

This work was supported by grants from the Spanish Ministerio de Sanidad, ISCIII (R006/009, Red Heracles), Ministerio de Ciencia e Innovación (BFU2007-61524 and BFU2010-15898) and Generalitat de Catalunya (CIDEM-VALTEC09-1-0042).
Investigation of the role of icilin, a TRPM8 channel agonist, in mediation of detrusor contraction in pig urinary bladder

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Transient receptor potential (TRP) channels have been shown to play an important role in the modulation of bladder sensation and afferent nerve activity. Recently a novel receptor, TRP melastatin 8 (TRPM8), activated by icilin and cool temperatures (8-28°C) has been identified in human bladders (1). The physiological and pathophysiological role of these channels remains unclear and few studies have investigated their function in mediating detrusor contraction (2). The aim of this study was to investigate the effect of icilin, a TRPM8 channel agonist, on strip and whole pig bladder contraction. Fresh female pig bladders were obtained from the local abattoir. For whole organ experiments, the bladder and the associated vasculature were excised and maintained under controlled physiological conditions, perfused with Krebs buffer. The effect of intravesical (IVE) or intravascular (IVA) administration of icilin (50μM) on carbachol-induced (10μM) whole bladder contractions was monitored by recording the IVE pressure (cmH2O). For isolated strip experiments, longitudinal strips of denuded detrusor or mucosa were mounted in Per- splex microbaths, superfused with Krebs solution and main- tained at 37°C. Strips were exposed to 10μM carbachol and once contractions had stabilised, 50μM icilin or vehicle were applied to the strips 10min before application of carbachol. All data are expressed as the mean±SEM. Statistical analysis was carried out by using repeated measure ANOVA followed by Dunnett’s post hoc test.

IVA administration of 50μM icilin significantly (p<0.05) decreased the magnitude of the carbachol-induced whole bladder (n=4) contraction as evidenced by reduced intravesical pressure rises (no icilin: 11.39±0.87 cmH2O vs. 50μM icilin: 9.60±1.12 cmH2O). In contrast, IVE administration (n=3) of 50μM icilin significantly (p<0.05) increased the carbachol-induced IVE pressure rise (no icilin: 13.71±2.05 cmH2O vs. 50μM icilin: 18.08±2.94 cmH2O). The carbachol-induced contractions of both detrusor (n=13) and mucosal (n=10) strips were also significantly inhibited by 50μM icilin (p<0.01 and p<0.05 respectively). The vehicle control had no effect on car- bachol stimulated whole bladder or muscle strip contractions at all concentrations.

Icilin appears to have the potential to modulate pig bladder contractility. Functional activation of TRPM8 channels in the whole organ resulted in different contractile responses depending on the route of icilin administration (IVE vs. IVA). However, consistent inhibitory responses were seen when strips of tissue (muscle & mucosa) were used. The differing smooth muscle responses seen with various routes of icilin administration may reflect regionalisation of mechanisms in different layers of the bladder wall.


Structure-function studies of the pore domain of the TMEM16 family of Ca2+-activated Cl- channels

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TMEM16 channels are a newly described family of anion channels unrelated in primary sequence to any previously described channel protein (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Recent studies have shown that TMEM16A and the related TMEM16B mediate many CaCC functions, including salivary exocrine and tracheal secretion, and oofactory transduction (Ferrera et al., 2010). The molecular features involved in controlling chloride conduction in TMEM16 channels, however, are still poorly understood. Hydropathy analysis of the primary sequence of TMEM16A indicates the presence of a re-entrant loop between the transmembrane domains 5 and 6. It has been suggested that the channel selectivity filter and the conduction pathway for anions reside within this area. To examine the functional role of this region, we used a chimeric strategy involving various TMEM16x proteins with different conduction properties.

Wild-type (wt) or mutant (chimeric) TMEM16x channels were heterologously expressed in HEK-293T cells and macroscopic currents were recorded with the whole-cell configuration of the patch-clamp technique. The external solution contained (mM): 130 CsCl, 10 glucose and 10 Hepes; pH was 7.4. The pipette solution contained (mM): 150 NaCl, 0.1 CaCl2, 10 glucose and 10 Hepes; pH was 7.7. Two-tailed t-tests were used for comparisons. Shifts in the reversal potential of the current caused by replacement of external Cl- with other anions gave the following selectivity sequence for TMEM16A channels based on permeability ratios (P(x)/P(Cl)):

- SCN- (6.0 ± 0.9) > ClO4- (4.6 ± 0.3) > I- (2.8 ± 0.2) > NO3- (2.4 ± 0.1) > N3- (1.9 ± 0.2) > Cl- (1.0) >> gluconate (0.12 ± 0.04) (n=4-7). The relative conductance was: N3- (8.9 ± 2.4) > SCN (4.0 ± 0.7) > ClO4- (3.5 ± 0.6) > I- (1.6 ± 0.2) > Cl- (1.0) >> gluconate (0.23 ± 0.03) (n=4-7). The relative anion conductance and selectivity sequences for TMEM16B channels were not statistically different from those of TMEM16A. Replacement of TMEM16A re-entrant loop (599-705) with the putative pore loop of TMEM16B gave rise to functional channels with the same degree of selectivity for anions as in TMEM16A channels. Chimeric channels obtained by substituting the re-entrant loop of TMEM16A with the re-entrant loop of TMEM16 proteins with very limited conductance for anions (TMEM16F, TMEM16K) resulted in channels unable to conduct anions. Taken together, these data reinforce the idea that the re-entrant loop contributes to the channel pore. It is likely that the regions within the sequence of the re-entrant loop that are identical between the TMEM16A and TMEM16B channel (599-607, 617-647, 694-705) contain the structural elements involved in controlling anion selectivity.


Rett syndrome (RTT) is an autistic spectrum disorder caused by mutations in the X-linked gene that encodes the transcription factor methyl-CpG-binding protein 2 (Mecp2). It features respiratory disorders with frequent apnoeas and periodic breathing (Weese-Meyer et al. 2006). The respiratory arrhythmia was successfully reproduced in heterozygous female mice (Mecp2+/−) with deletion of the 3rd and 4th exons (Bissonnette and Knopp, 2008). We demonstrated that augmenting endogenous GABA in the brainstem of Mecp2+/− females markedly reduces apnoeas (Abdala et al. 2010), but the mechanisms have not been fully determined. Since null male mice have prolonged post-inspiratory neuronal activity during apnoeas (Abdala et al. 2010; Stettner et al. 2007), we hypothesized that insufficient GABAA inhibition in the Kölliker-Fuse (KF) nucleus, a key control region of post-inspiratory (post-I) phase duration, underlies these respiratory disorders.

All procedures conformed to the UK Home Office guidelines on animals (Scientific Procedures) Act 1986 and were approved by the University of Bristol’s Animal Ethics Committee. Heterozygous females (Mecp2+/−) (n=5) were deeply anaesthetized with 5% halothane and decerebrated. We used an in situ arterially perfused preparation to record phrenic, central vagal (for measurement of post-I) and hypoglossal nerve activity (Paton, 1996). We used a three-barrelled microinjection pipette (<20 μm o.d.) containing glutamate, NO-711 (a GABA reuptake blocker) or fluorescent beads diluted in aCSF in each barrel. Glutamate microinjections helped functional location of the KF nucleus prior to drug injections. Sites were marked with fluorescent beads.

In the MeCP2+/− mice NO-711 (10 mM, 60 nl, unilateral) microinjection into KF nucleus decreased the duration of post-I activity to 57±8% (mean ± SEM, t test, P<0.05) of the pre-injection level. In addition, NO-711 reduced the coefficient of variability for post-I from 0.82 ± 0.15 (n=10, CHU: 1.8%, P=0.012 Student’s unpaired t-test).

In conclusion, CHU-induced hyperinnervation of the tibial artery (that supplies muscles of the rat hindlimb) persists into adulthood. However, this does not appear to have gross systemic cardiovascular consequences at this age. Further work will determine the sensitivity to, and the levels of sympathetic nerve activity present in the muscle vasculature.

Abdala APL et al. (2010) PNAS, 107(42), 18208–18213.


PC124

The effects of the diving reflex on cerebral blood flow whilst supine with and without mild heat stress: A pilot study
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Heat stress, even when mild, decreases cerebral blood flow (CBF) and thus compromises orthostatic tolerance (OT). Whole-body skin cooling has been shown to attenuate the reduction in CBF during heat stress and improve OT. Forehead-cooling (FC) has also been shown to increase CBF; due, in part, to sympathetic mediated vasoconstriction of the peripheral vasculature (the ‘diving reflex’). However, it is currently unknown whether FC can attenuate the decrease in CBF in response to mild heat stress. In a cross-over design, 8 healthy males completed trials in both 20oC and 40oC. Whilst supine, mean arterial pressure (MAP, Finometer), heart rate (HR), middle cerebral artery blood flow velocity (MCAv, Doppler), ventilation, end-tidal PCO2 (PETCO2), weighted mean skin (TSK) and rectal (TC) temperatures were measured continuously. Cardiac output (Q) was also calculated (Model Flow method). Baseline measures were recorded after 20 minutes of resting supine and 1 min prior to cooling. Cooling was applied via an ice pack (0oC) to the forehead for 2 minutes. Baseline values and percentage change data were averaged over 1 min. FC was effective in decreasing temperature of the head by 8±2 oC. TC was unchanged by heat stress and FC. TSK was greater in 40oC (P<0.01), however FC reduced TSK (P=0.046) from baseline. Heat stress elevated HR (14±6%, P<0.001), Q (26±14%, P=0.008) and decreased MAP (-9±7%, P=0.022). FC tended to elevate MCAv (5 ± 7%, P=0.085) from baseline during heat stress. MCAv was unaffected by FC at 20oC (P>0.05), however, FC tended to decrease HR (P=1.54) from baseline values. MAP, Q, PETCO2 and VE were unchanged (all P>0.05) from baseline by FC in both ambient conditions. In summary, we observed a stereotypical cardiovascular response to heat stress and an, on average, increase in MCAv during face cooling. These pilot data show the potential of face cooling as a means to improve OT during heat stress. Further study is needed to confirm these observations.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC125

Age-dependent effects on atrial arrhythmogenicity in Scn5a+/- murine hearts
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Recent clinical studies associate both ageing and mutations in the gene encoding the cardiac Na+ channel, SCNS5A, with atrial arrhythmogenicity. Atrial action potential initiation, propagation and recovery were compared in young (3 month) and aged (12 month), wild-type (WT) and loss-of-function Scn5a+/- murine hearts previously used to model Brugada Syndrome. Multi-electrode array recordings assessed the spatial propagation and duration of intrinsic electrical activity in superfused atrial preparations. Bipolar electrogram recordings (BEGs) measured basic cycle lengths (BCLs) in Langendorff preparations. Durations of electrogram activity (EGDz), during regular (S1) and extrasystolic (S2) stimulation during programmed electrical stimulation (PES), provided both EGD ratios and atrial effective refractory periods (AERPz). Finally, monophasic recordings measured action potential durations (APDs). Systematic statistical explorations for independent and interacting effects of age and the Scn5a+/- condition demonstrated that young and aged Scn5a+/- mice showed increased intrinsic BCLs as well as slowed propagation of atrial excitation relative to the corresponding WT with the greatest effects in the aged Scn5a+/-.. However, the aged Scn5a+/- showed normal APDs, EGDs and EGD ratios, increased AERPz, and smaller APD/AERP ratios. In contrast, the young Scn5a+/- showed prolonged electrograms and APDs as well as greater EGD and APD/AERP ratios, measures previously used to assess arrhythmogenicity. In conclusion the Scn5a+/- condition exerts effects upon measures of atrial arrhythmogenicity that overlap with those recently described for the gain-of-function Scn5a+/+KQPQ condition, but which produce their maximum effects in young rather than aged animals.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC126

A comparison between standard and dynamic electrical restitution in single cells
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Electrical restitution (RT - the relationship between action potential duration [APD] and diastolic interval [DI]), is a key determinant of ventricular fibrillation (VF) initiation. Whilst restitution is usually studied in whole hearts, its investigation in single cells is relatively unexplored. There are two main methods to measure electrical restitution – standard (STRT) and dynamic restitution (DYRT). Our aim is to compare STRT and DYRT in single cells. Adult NZW rabbits (n=3) were anaesthetised with propofol (10mg/kg, IV) and euvanized with an overdose of pentobarbitone (IV). Hearts were isolated and standard digestion techniques used to obtain myocytes from left ventricular base. Action potentials were recorded (33-35oC) in current-clamp mode using the whole cell configuration of the patch-clamp technique. STRT was obtained using a single extra-stimulus protocol, where cells were paced at 400ms cycle length (CL, 20 beats [S1]) followed by an ‘S2’ stimulus at programmed intervals down to effective refractory period (ERP – the longest CL that failed to capture). DYRT data was obtained by constant pacing for 100 beats from 350ms to ERP. RT curves were plotted and the slopes analysed. Maximal slope, D1/slope = 1, DI range for slope<1 (DI range), APDmax, slope at DI of 30ms, 50ms and 90ms (sRT30, sRT50, sRT90 respectively). For additional analysis of DYRT, APD alternans developed (APD differ-
ence >5ms) and were quantified, including the CL at which alternans occurred; maximum amplitude of alternans, the CL range that alternans occurred and sum of all alternans amplitudes (alternans index)). Data (mean±SEM) were analysed using paired students t-test, P<0.05 considered significant. Most analysed parameters were comparable (Table) between STRT and DYRT (n=5), but there was a trend towards a smaller ERP in DYRT. There were differences in the RT slopes (Figure) with sRT50 and sRT90 greater in DYRT compared with STRT. This is the first detailed study of restitution parameters in isolated ventricular myocytes. Parameters from STRT and DYRT are comparable to those from whole hearts. Noticeable differences exist with STRT curves following a mono-exponential curve, whereas dynamic RT is more linear. The key outcome is that there is comparable and quantifiable data so that single cells can be used to determine the cellular mechanisms behind arrhythmias generation.

<table>
<thead>
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</tbody>
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Table

Koller et al, AJP, 1998. 275;H1635

The Garfield Weston Trust. The study is part of the research portfolio supported by the Leicester NIHR Biomedical Research Unit in Cardiovascular Disease.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC127

**Structured Culture Scaffolds force the Maturation of Calcium Transients in Rat Neonatal Ventricular Myocytes**

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Background: Cardiomyocytes differentiated from pluripotent stem cells and neonatal rat ventricular myocytes (NRVM) are widely advocated as models to study the physiology, pathophyology and pharmacology of the myocardium in vitro. The application of these cell lines is however limited by their immature phenotype which is poorly representative of adult myocardium. For this reason we aim to investigate the potential of structured culture scaffolds which force the alignment of myocytes on calcium transients, an important aspect of myocyte physiology.

Methods: Two structured constructs were fabricated as previously described in the literature: microgrooved polydimethylsiloxane (PDMS) scaffolds [1-3] and micropatterned fibronectin on flat PDMS membranes [1,2] (Figure 1). NRVMs were isolated using a method described previously and with complete compliance to Home Office guidance [4]. NRVMs were then seeded at a density of 2 million cells per 60mm culture dish containing micropatterned PDMS or 2/3 million cells per well in a 12 well plate containing PDMS scaffolds. After 24 hours preparations were washed with prewarmed DMEM and complete medium to remove dead and non-adherent cells [4]. To visualise Ca2+ transients NRVMs were loaded with Rhod-2 AM (60 μM, Invitrogen) in DMEM at 37°C for 30 minutes, then washed and incubated in prewarmed DMEM with 2% FBS (Invitrogen) for 30 minutes to de-esterify. Rhod-2 AM was excited at 552-nm, and the emitted fluorescence was collected through a 581-nm long-pass filter. Confocal line scanning was performed (LSM 510, Carl Zeiss Micro Imaging) whilst NRVMs were beating spontaneously and whilst field stimulated at 0.5Hz, 1Hz and 2Hz. Time-to-peak was taken as the time taken for the ratio signal to go from baseline fluorescence to peak fluorescence. 50% and 90% decay were the time taken for the Rhod-2 AM Ca2+ transient to decline by 50% or 90% of the transient amplitude from peak fluorescence [5].

Results The mean time-to-peak was significantly shorter in the Structured Constructs compared with control cells (P<0.0005, Mann Whitney Test) as was the time to 50% (P<0.0005, Mann Whitney Test) and 90% decay (P<0.005, Mann Whitney Test) when the RNVM were paced at 1Hz (Figure 2). Similar findings were seen when the RNVM were paced at 1Hz and when the cells beat spontaneously (Figure 2). This trend was not seen at 2Hz pacing in any group as there was not sufficient time between stimulation for the Ca2+ transients to return to baseline.

Conclusion: Structured culture scaffolds affect Ca cycling of RVNM calcium transients. The mechanisms underlying these observations need to be elucidated and further characterisation of the effect of structured culture scaffolds and other aspects of cardiomyocyte electrophysiology is required.

**Figure 1.** AJRVNM on Micropatterning of the PDMS membrane. BJKVNM on Microgrooved PDMS scaffolds. C) Unstructured RVNM. D) Immunohistochmetry of RVNM on Microgrooved PDMS scaffolds.
In atrial function in age, we have developed a conditional knock-out (CKO) mouse model where a central component of this pathway: MKK4, has been specifically deleted from the atria (De Lange et al. 2003; Liu et al. 2009). Hearts were assessed for atrial arrhythmia, both in vivo (using ECGs performed under 2% inhalation of isoflurane anaesthetic) and ex vivo at 3 and 12 months of age (n=6 per group) and tissue was collected for molecular analysis.

Surface ECGs of these mice at 3 months of age showed abnormal atrial excitation with reduced P amplitudes (control 0.084mV ± 0.01 vs. CKO 0.058mV± 0.01 t-test p<0.05; mean ± SEM) but no arrhythmia; however as the mice aged they became more susceptible to atrial arrhythmia, such as atrial tachycardia (AT) and atrial ectopic beats. Ex vivo hearts from 12 month old MKK4 CKO mice were more likely to develop AF/AT with electrical programme stimulation than old control mice (37% control mice vs. 100% CKO mice). Ex vivo conduction mapping revealed longer atrial conduction times in these mice, which could make the hearts more vulnerable to re-entry arrhythmias. In old CKO mice an increase in fibrosis was detected by picro-sirius red stain of tissue sections (2.8% ± 0.4 in control vs. 6.1% ± 0.4 in CKO t-test p=0.01; mean ±SEM). The expression of fibrotic pathway components was assessed using real-time PCR and western blot analysis and CKO mice had altered patterns of expression of TGF-β1, TGF-β receptors 1 and 2, MMP2/9 and TIMP2, compared to control mice. Development of an atrial specific MKK4 knockout mouse therefore reveals a role for the MAPK pathway in protecting against atrial arrhythmia in age through regulation of fibrotic processes and could provide insight into the design of more targeted therapies for atrial fibrillation.

Lui W et al. (2011) J Mol Cell Cardiol. 50:702-711

This work is supported by The Wellcome Trust and The British Heart Foundation.

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PC128

Increase in atrial arrhythmic susceptibility with age in mice with atrial-specific MKK4 deletion

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Cardiovascular disease is the most common cause of death in Western countries and 50% of mortalities attributable to cardiac causes are accounted for by cardiac arrhythmias. Atrial fibrillation (AF) is the most common form of sustained arrhythmia, its incidence increases with age and it is associated with extensive structural, contractile, and electrophysiological remodelling. Current pharmacological treatment of AF is limited and understanding the molecular mechanism of such remodelling processes is essential for the development of new targeted therapeutic interventions. A pathway that is potentially involved in regulating the pathogenesis of AF is the mitogen activated protein kinase cascade (MAPK), since components of the MAPK pathway have been shown to be down-regulated in patients with permanent atrial fibrillation (Kartmann et al. 2005). The MAPK pathway is involved in the regulation of fibrosis in the ventricle in aged/hypertrophied hearts (Lui et al. 2011; Kyo S et al. 2006) and fibrosis is linked to atrial fibrillation. To establish the role of the MAPK pathway in atrial function in age, we have developed a conditional knock-out (CKO) mouse model where a central component of this pathway: MKK4, has been specifically deleted from the atria (De Lange et al. 2003; Liu et al. 2009). Hearts were assessed for atrial arrhythmia, both in vivo (using ECGs performed under 2% inhalation of isoflurane anaesthetic) and ex vivo at 3 and 12 months of age (n=6 per group) and tissue was collected for molecular analysis.

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PC129

Structural and Functional Remodelling Both Contribute to Arrhythmia Substrate in Computational Models of Right Heart Failure

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Right heart failure (RHF) can lead to an increased risk of arrhythmias, yet the mechanisms underlying these arrhythmias are less well understood than those in left heart failure (Voelkel et al., 2006). We have developed a computational model of rat RHF, based on experimental measures from monocrotaline (MCT) injected rats, to investigate structural (anatomical) and functional (electrophysiological) mechanisms underlying re-entrant arrhythmias. Ion channel mRNA and monophasic action potential data obtained from both the left (LV) and right (RV) ventricles of control and failing (60 mg/kg MCT) rats (Benoist et al., 2011)
were used to scale ion channel conductances in the Pandit et al. (2001) single cell rat model. These were then incorporated into 1D heterogeneous tissue strands, and heterogeneous and anisotropic 3D ventricle models (3 control, 3 RHF). For the ventricle models, anatomy was reconstructed from diffusion tensor magnetic resonance imaging (DT-MRI) at 200 \( \mu \)m resolution (Benson et al., 2011).

Model cellular action potential duration increased in RHF, from 36 to 51 ms in LV epicardial cells and from 31 to 78 ms in RV cells, consistent with experimental recordings. In 1D tissue models, RHF increased the refractory period in both the LV and RV, while the mean temporal width of the vulnerable window for unidirectional conduction block increased with RHF in the LV, from 0.8 to 1.4 ms, but decreased in the RV, from 2.2 to 1.7 ms. In the 3D DT-MRI reconstructions, there were no changes to LV structure with MCT, but mean RV wall thickness increased from 1.6 to 2.2 mm and the mean rate of transmural fibre rotation decreased from 148 to 86 \( ^{\circ} \)/mm. Following programmed stimulation in the RV wall, we found an increased propensity for sustained arrhythmias in RHF, with the structural and functional changes playing a synergistic role in this increase.

In conclusion, using a hierarchy of rat ventricular tissue models we have shown that the initiation and maintenance of arrhythmias in RHF is dependent on both structural and functional remodelling.


Funded by the Medical Research Council and the British Heart Foundation.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC130

Depressed TS-evoked glutamatergic transmission on NTS neurons of CIH rats is due to reduced number of functional synapses

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Chronic intermittent hypoxia (CIH) induces several changes in the cardiovascular and respiratory neural control, including reduction in the afferent synaptic transmission on nucleus tractus solitarii (NTS) neurons. Here we studied the mechanisms involved in this reduction in NTS neurons of CIH rats. Male rats (21 days old) were exposed to 10 days of CIH, and after this period whole-cell glutamatergic synaptic currents were recorded in neurons within caudal and intermediate NTS in horizontal brainstem slices at room temperature. Tractus Solitarii (TS)-evoked glutamatergic post-synaptic currents (eEPSCs) were obtained by TS electrical stimulation in the presence of GABA\(_A\) receptor antagonist bicuculline. The neurons were classified according to the standard deviation (SD) of latency for eEPSCs, as 2nd-order neurons (latency SD<250 \( \mu \)s) and putative higher-order neurons (latency SD>250 \( \mu \)s). CIH selectively reduced the amplitude of eEPSCs in 2nd-order neurons (325\( \pm \)22 vs. 187\( \pm \)10 pA, n=58; \( P<0.0001 \)) but did not affect the eEPSC amplitudes of putative higher-order NTS neurons (102\( \pm \)19 vs. 113\( \pm \)16 pA, n=20). Concerning the mechanisms of eEPSC reduction in 2nd-order neurons CIH did not affect the short-term depression of the non-NMDA eEPSCs, suggesting the absence of changes in release probability. This evidence is supported by the lack of changes in mEPSC frequency as well as in the number of failures of eEPSCs evoked in cadmium (Cd\(^{2+}\)) or low calcium experiments (0.2 mM). In addition, a post-synaptic effect of CIH is ruled out because the half-width of eEPSCs, the amplitude of mEPSCs and the amplitude of eEPSCs evoked in the presence of low Ca\(^{2+}\), Cd\(^{2+}\) or strontium [Sr\(^{2+}\) (asynchronous eEPSCs)] were similar in both groups. However, the total number of asynchronous eEPSCs recorded in the presence of 2 mM of Sr\(^{2+}\) was significantly reduced after CIH suggesting a decrease in the number of functional synapses. A minimal stimulation protocol of the TS showed that the amplitude of the putative single-fiber eEPSCs was slightly diminished after CIH (176\( \pm \)0.6 vs. 162\( \pm \)0.5 pA, n=22, \( P<0.05 \)) while the estimated number of afferent fibers was greatly reduced (24\( \pm \)2 vs. 12\( \pm \)1 fibers, n=22, \( P<0.0001 \)). These findings support the concept that CIH selectively depresses TS-evoked glutamatergic synaptic transmission on 2nd-order NTS neurons by a reduction in the number of functional synapses, probably due to a plastic homeostatic adaptation to the overexcitation of visceral afferents inputs in CIH rats.

Supported by FAPESP and CNPq.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC131

Angiotensin-II-dependent renovascular hypertension and cardiac dysfunction are improved by regular swimming exercise: role of the oxidant-antioxidant balance

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Renovascular hypertension (RVH) remains among the most prevalent causes of secondary hypertension, which develops from activation of the renin-angiotensin system and recruitment of the oxidative stress pathways (Textor, 2009). The impact of physical activity in prevention of renovascular hypertension and oxidative stress has not been elucidated yet. In the present study, the potential protective and therapeutic effects of moderate exercise training (30 min/day 5 days/week swimming for 9 weeks) on the cardiac tissues of Wistar albino rats with experimental RVH were determined. Under ketamine and chlorpromazine (100 and 2 mg/kg, respectively, i.p.) anesthesia 2-kidney-1-clipping hypertension was induced as a RVH model with elevated circulating levels of Angiotensin-II. Rats (10-week-old, male, n=55) were divided into 4 groups: sham-operated control, sedentary RVH, post-surgery trained RVH and pre-surgery trained RVH. Echocardiographic imagings were made and the arterial blood pressure (BP) measurements were recorded by tail-cuff method. Rats were decapitated and trunk blood was obtained for the measurement of serum levels of IL-2, IL-6 and TNF-\(\alpha\), while malondialdehyde (MDA), glutathione (GSH) and catalase (CAT) levels and myeloperoxidase (MPO) activity were studied in the cardiac tissue samples. Values are means ±
S.E.M., compared by ANOVA. RVH in the sedentary group (173±4.9 mmHg) resulted in increased BP with respect to control group (128±2.1 mmHg; p<0.001). Pre-surgery training prevented the elevation in BP (139±1.4 mmHg; p<0.001), while exercise after the RVH did not alter BP significantly (174±2.8 mmHg). Increased left-ventricular systolic diameter in the sedentary RVH group (4.97±2.3 mm) was decreased in both trained groups (pre-surgery: 3.61±0.19 mm; p<0.05, post-surgery: 3.22±0.17; p<0.01) with respect to control (2.73±0.21 mm; p<0.001). The increased levels of serum IL-2, IL-6, TNF-α levels in the sedentary group as compared with the control group (p<0.05) were significantly decreased in pre-surgery trained RVH group (p<0.01). Cardiac MDA levels and MPO activities were increased in the non-exercised RVH group, while training before or after the surgery abolished these elevations (p<0.01). Similarly, depleted GSH and CAT levels in the cardiac tissues of sedentary RVH rats were found to be preserved in both means of exercise (p<0.001). Current results demonstrate that moderate training improves ventricular functions, controls high blood pressure in RVH, while RVH-induced oxidative damage in cardiac tissue is ameliorated through the modulation of oxidant-antioxidant balance. In conclusion, exercise does not only improve the cardiac and circulatory functions, but it also initiates an anti-inflammatory process to defend against the angiotensin-II-induced cardiac injury.

Supported by MU Scientific Research Projects Commission. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC132

Is cerebral blood flow a determinant of a long term arterial pressure set-point in the rat?

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Cushing (1901) discovered that acute increases in cerebral vascular resistance produced rises in arterial pressure. Subsequently, Dickinson and Thompson (1960) discovered that antemortem blood pressure correlated with a narrowing of the vertebral arteries such that hypertensive subjects had stenosed vessels; carotid and renal arteries did not show this correlation to be so strong. An explanation advanced by these authors was that restricted blood-carrying capacity of the vertebral arteries caused high blood pressure, but the issue of causality has remained controversial ever since. In the present study, we have sought to determine what effect reducing cerebral blood flow has on the chronic regulation of arterial pressure in the normotensive rat.

All procedures were approved by the University of Auckland ethics committee. Under deep Halothane anaesthesia (3%; assessed by absence of pinch reflex to paw or the tail) Wistar rats were implanted with telemeters (Telemetry Research Ltd) for measuring arterial blood pressure chronically. Basal levels were monitored for 9 days prior to occlusion of both vertebral arteries, or both vertebral arteries plus a common carotid artery or both vertebral arteries, a common carotid artery and a common carotid artery clip to reduce blood flow. In rats in which common carotid arteries were ligated or partially occluded, the carotid sinus baroreceptors were denervated (under Halothane anaesthesia as above). Arterial pressure was monitored for up to 4 weeks. Bilateral occlusion of vertebral arteries alone was without effect on systolic blood pressure (SBP) or heart rate (n=3). In contrast, SBP was elevated in rats in which either both vertebral arteries plus a common carotid artery were occluded or occlusion of vertebral arteries, a common carotid artery and clipping the remaining common carotid artery. There was no difference in the pressor effect between these two latter groups which comprised an initial rise of 15±2 mmHg (mean±SEM; n=5, P<0.05 t-test). Over the following week there was a gradual and partial return towards control levels but a pressor effect remained above control levels (5±0.5 mmHg) that persisted for >20 days. In addition, the diurnal variability of blood pressure was enhanced. Post hoc analysis of the cerebral circulation indicated marked compensation including dilatation of the posterior communicating arteries and ventral spinal arteries feeding into the vertebrobasilar circulation.

These studies provide the first proof of principle that reductions in cerebral blood flow can elevate arterial pressure chronically. Our findings are consistent with the notion of an intracranial baroreceptor (Rodbard & Stone, 1955). Our data also indicate that there is an impressive ability to compensate for reduced cerebral blood flow by recruitment from arteries in the spinal cord.


JFRP was in receipt of a Royal Society Wolfson Research Merit Award. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC133

Reduction in blood flow to a localized region of the dorsomedial medulla triggers hypertension in rats

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Our hypothesis is that cerebral blood flow is a major long term determinant of the set point of arterial pressure. The brain-stem nucleus of the solitary tract (NTS) is a pivotal region for regulating the set-point of arterial pressure (Doba & Reis, 19730; Duale et al. 2007), the mechanisms of which are not fully understood. Based on evidence that the NTS exhibits O2-sensing mechanisms (Lo et al. 2006), we examined whether a localized reduction of blood supply, resulting in stagnant hypoxia in the NTS, would lead to an increase in arterial pressure.

Experiments were approved by the Ethics Committee for Animal Experiments at Wakayama Medical University, Japan. Male Wistar rats were anesthetized with urethane (1.45 g/kg i.p.) and the level of anaesthesia was monitored by assessing a limb withdrawal response to a noxious pinch. Supplements of urethane (0.145 g/kg, i.p.) were given as required. Cardiovascular parameters were measured before and after specific branches of superficial dorsal medullary veins were occluded using microclips at a level ±0.5 mm rostral/caudal to the calamus scriptorius; we assumed these were drainage vessels from the NTS and would produce stagnant hypoxia. Some rats were sino-aortic nerve denervated (SAD). Tissue blood flow was...
measured using a laser flow meter (Advance Co., Japan; 0.25 mm diameter) inserted into the dorsomedial medulla. Hypoxprobe-1 - a marker for detecting cellular hypoxia in the post mortem tissue, confirm whether vessel occlusion induced hypoxia and if this included the NTS.

Following vessel occlusion, blood flow in the dorsomedial medulla showed a ~60% decrease (resting level: mean±SEM, 49±4 ml/min/100g tissue; n=6, t-test p<0.001) and was associated with hypoxia in neurons located predominantly in the caudal and intermediate part of the NTS as revealed using hypoxprobe-1 immunoreactivity. Vessel occlusion induced an immediate increase in arterial pressure in both intact (from 87±3 to 101±5 mmHg; n=9, P<0.05) and SAD rats (from 91±2 to 116±3 mmHg; n=11, P<0.05). The pressor response was greatest in SAD rats (P<0.01). In SAD rats this response persisted for >1 hour whereas in intact rats only 30 min.

This study demonstrates that reduced blood flow and localized hypoxia in the NTS region increases a prompt rise in arterial pressure; the magnitude of the response is blunted by arterial baroreceptors. We suggest this response is a protective mechanism whereby the elevated systemic pressure is a compensatory reaction to enhance cerebral perfusion. Whether this mechanism has any relevance to the aetiology of neurogenic hypertension is unknown.


Japan Society for the Promotion of Science (21300253, 19599022 and 19-07458), the Takeda Science Foundation and British Heart Foundation funded research. JFRP was in receipt from the British Heart Foundation of a Royal Society Wolfson Research merit Award

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC134

Electrical restitution in single cells - effects of acetylcholine and nitric oxide donors

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Introduction

Electrical restitution (RT: the relationship between action potential duration [APD] and diastolic interval [DI]), is a key determinant of ventricular fibrillation initiation. Steep RT slopes confer an increased risk of arrhythmia. We have shown that vagus nerve stimulation (VNS) is protective against VF via a flattening in the RT slope, an effect mediated by nitric oxide (NO)[1]. The cellular mechanisms and signaling pathways of this effect are unknown. Our aim was to study RT in single cells and investigate the effect of acetylcholine (ACH) and exogenous NO using the NO donor SNAP.

Methods

Left ventricular base myocytes were isolated using standard techniques from adult NZW rabbits. Animals were sedated with propofol (10mg/kg IV) and euthanized with an overdose of sodium pentobarbitone (IV). Action potentials were recorded at 33-35°C in current-clamp mode using the whole cell configuration of the patch-clamp technique. RT data were obtained using a single extra-stimulus (S1-S2) protocol. RT curves were plotted and the effect of ACH (1µM) and SNAP (100µM) on maximal RT slope and APD determined. The effect of inhibiting soluble guanylyl cyclase (sGC) with ODQ (100µM) was also investigated. Effective refractory period (ERP), APD90 during constant pacing (APDCP), maximum RT slope and maximal APD90 during RT (APD90MAX) were the electrophysiological parameters measured. Data (mean±SEM) were compared using student’s paired t-test. *P<0.05, **P<0.01, NS P>0.05.

Results

ACH (n=4-6) and SNAP (n=7-11) had no significant effect on ERP or RT slope. Whilst ACH and SNAP had no effect on APDCP at 2.5 Hz, SNAP significantly prolonged APD90MAX. ODQ not only reversed the effect of SNAP, but caused a profound further shortening at all DIs. Preliminary results (n=2) indicate that application of ODQ alone also causes large decreases in ERP, APDCP and APD90MAX. Mean results are shown in the table below. 1BL vs SNAP, 2BL vs SNAP+ODQ, 3SNAP vs SNAP+ODQ.

Conclusion

ACH had no significant effects on electrical restitution in single rabbit ventricular cells and maximum RT slope was unaffected by any of the investigated reagents. Although SNAP had no effect on APDCP, it significantly increased APD90MAX, a result similar to that observed in isolated whole heart experiments. This suggests that NO has dynamic, rate dependent effects on cardiac ionic currents. Our results are consistent with this effect of exogenous NO being mediated by a cGMP dependent pathway and also with electrophysiological effects from basal levels of sGC activity under our conditions.

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Fig 1: A) Effect of ACH on RT. B) Effect of SNAP and ODQ on RT


Garfield Weston Trust, Biomedical Research Unit, Leicester.

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Poster Communications

PC135

Right atrioventricular ring tissue in human embryonic hearts – what can be learnt from histological and immunohistochemical investigation?

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The cardiac conduction system (CCS) comprises specialized myocytes which have the ability to elicit an action potential and then conduct it from the atria to the ventricles. This system includes the sinus node, atrioventricular node, His bundle and Purkinje fibres. In the early 20th century principles were set regarding histological characterisation of conducting myocytes. Three criteria had to be met for cells to be accepted as part of the CCS; they had to be histologically distinct, serially detectable from section to section and encapsulated from surrounding myocytes by fibrous tissue (1, 2). The generation of improved immunohistochemical methods allows the morphology of the CCS to be highlighted more accurately. These methods have enabled the discovery of other nodal-like tissue, such as the atrioventricular ring tissue (AVRT). Cells of the AVRT encircle the tricuspid, mitral and aortic valves and form the right, left and aortic rings. These three rings combine to produce the retroaortic node. The function of the AVRT remains to be defined although it has been demonstrated that catheter ablation of this region attenuates atrial tachycardias. The aim of this pilot study was to determine if atrioventricular ring tissue is present in human embryos at the stage when the heart is fully developed, using markers, such as, HCN4, Cx40 and Cx43. 4 embryonic hearts at the fully developed stage (youngest heart at Carnegie stage 23) were frozen and cryosectioned in the longitudinal axis from the dorsal to the ventral surface to give tissue sections 25 μm in thickness. Histology was carried out on sections (every 300 μm) in order to find the approximate location of the ring tissues within the heart. Adjacent sections to those used for histology were immunolabelled for HCN4 (marker of the CCS and AVRT), Cx40 (marker of the atrial myocardium) and Cx43 (marker of the working myocardium). Since, HCN4 was found to be a non-specific marker in human embryos, attention was drawn to Cx40 and Cx43. The antibody concentration was optimised (Cx40, 1:50, Cx43, 1:50) so that atrial and ventricular myocardium could be easily identified. The results showed that Cx40 is present in the atrial myocardium and Cx43 is present in the working myocardium. Myocytes of the right atrioventricular ring tissue did not show either Cx40 or Cx43 to be present in this region. Our findings indicate that specialized atrioventricular ring tissues may be present in the human embryonic hearts, which can explain atrial tachycardias arising in the adult heart, in this region. We will now carry out further investigations in order to determine the expression of different proteins in the right ring tissue. We will also establish the relationship of the human embryonic AVRT to the CCS and determine whether it is similar to that described in the rat model.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC136

Effects of exercise training in intraplatelet L-arginine-NO pathway and platelet aggregation in rats with myocardial infarction

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Introduction: Myocardial infarction (MI) is one of the major causes of heart failure and is associated with endothelium dysfunction and alterations in platelet activity in humans. Nitric oxide (NO) is a potent vasodilator that also inhibits platelet activation and aggregation. The purpose of this study was to assess the effect of exercise training on platelet aggregation and the L-arginine-NO pathway in an experimental model of MI.

Methods: 40 male Wistar rats (200-250 g) underwent anterior descending coronary artery ligation or sham surgery, previously anesthetized with tribromoethanol injected intraperitoneally (300mg/kg), resulting in four groups (n=10, each): MI/sedentary (MISed), MI/exercise (MIEx), Sham/sedentary (SSed), and Sham/exercise (SEX). The experimental procedures were approved by the Institutional Animal Care and Use Committee (CEA/051/2009) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After 8 weeks with 30 min of daily treadmill training (60% of maximum velocity), the animals were anesthetized with sodium thiopental (40 mg/kg, intraperitoneally) and the platelets were isolated from centrifugation of the blood collected from the abdominal aorta. Platelet L-arginine transport was assessed by incubation with L-[3H]-arginine (100 μM) and NOS activity, by the conversion of L-[3H]-arginine into L-[3H]-citrulline. Platelet aggregation induced by ADP (20 μM) was measured in whole blood by electrical impedance (560CA, Chronolog Corporation, PA, USA). Data were compared with a one-way ANOVA, and significance level was set at 5%.

Results: Exercise tolerance significantly improved (P < 0.05) in both MIEx and SEx after long-term physical training, as expected. However, no significant differences among the groups were noted in platelet aggregation, platelet L-arginine transport and NOS activity (Table 1).

Table 1. Platelet aggregation and L-arginine transport and nitric oxide synthase (NOS) activity.

Conclusion: Platelet function and the L-arginine-NO pathway were not affected by either MI or exercise training. It is plausible to assume that the experimental model of MI used in this study does not mimic the vascular alterations observed in human MI.
Platelet aggregation, L-arginine transport and nitric oxide synthase (NOS) activity

| Table 1: L-arginine transport across endothelial cell monolayers
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<td>L-arginine transport</td>
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This work was supported by FAPERJ and CNPq grants.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC137

The time-of-day variation in the response of ventricular myocytes to isoproterenol is lost in spontaneously hypertensive rats

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We have demonstrated a time-of-day variation in many key elements of E-C coupling in rat ventricular myocytes, including L-type Ca²⁺ current (LTCC), systolic [Ca²⁺], and SR Ca²⁺-loading, which appears to reduce the incidence of arrhythmias in response to isoproterenol (ISO) (1). In a pressure-overload induced model of left ventricular hypertrophy in rats, the normal cycling of the circadian clock controlled genes are severely blunted (2). We have looked at the impact of hypertension on the time-of-day variation in E-C coupling.

Adult male spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) and Wistar rats were housed with a 12 hour light/dark cycle. Single left ventricular myocytes were isolated at ZT3 the resting period or ZT15 the active period, where ZT0 refers to “lights on”, by enzymatic digestion of hearts excised at <6 weeks and >24 weeks. [Ca²⁺], was measured using Fura-2, LTCC using whole-cell patch clamp electrophysiology and NOS protein using Western blot analysis.

(Data are mean±SE; n=number of cells, hearts; one-way ANOVA with Bonferroni post-hoc test).

The data show a time-of-day variation in systolic [Ca²⁺], in ventricular myocytes from younger animals (<6 weeks) at 636±40nM vs. 498±19nM (n=21, 3; p<0.01) in WKY and 799±29nM vs. 554±20nM (n=23, 3; p<0.01) in SHR, for ZT3 versus ZT15 respectively. This variation persisted in older WKYs (>24 weeks) at 579±28 vs. 459±17nM (23.3; p<0.01) but was absent in older SHR at 666±33 vs. 669±31nM (21.3; ns). We also found a time-of-day variation in response to β-adrenoceptor stimulation with ISO, with a significant reduction in maximal systolic [Ca²⁺] recorded in 100nM ISO at ZT15 compared to ZT3 in ventricular myocytes isolated from younger WKY and SHR animals. However, this time-of-day variation whilst present in older WKY animals at 2,024±163 vs. 1,334±90nM (17.5; p<0.001) was absent in the older SHR at 1,984±175 vs. 1,742±104nM (20.5; ns). We have previously suggested that this time-of-day variation reflects higher levels of NOS-signalling in ZT15 animals, which depresses the response to ISO (1). Our data show low levels of nNOS expression in adult Wistar animals, which is up-regulated in SHR hearts (>24 weeks), but with no significant time-of-day variation. However, eNOS was detected at high levels in the adult Wistar hearts and showed significant time-of-day variation at 1.06±0.09 vs. 1.69±0.16 (eNOS)/β-Tubulin, n=6; p<0.05) for ZT3 versus ZT15 respectively. This variation was absent in SHR hearts at 1.08±0.23 vs. 1.07±0.11 (n=4; ns).

Our data show that the time-of-day variation in Ca²⁺-regulation during E-C coupling and modulation by ISO, is absent in older SHR hearts, which may have implications for sympathetic-induced arrhythmic activity.


HEC was supported by the department of Cardiovascular Sciences and HET by the BHF

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC138

Ischaemic preconditioning and remote ischaemic preconditioning modulate pre-ischaemic Ca²⁺ homeostasis in rat ventricular myocytes

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Ischaemic preconditioning (IPC), where the heart is subjected to one or more short periods of ischaemia prior to a longer period of index ischaemia, provides significant cardioprotec-

ion (1). More recently it has been demonstrated that preconditioning of organs or tissues remote from the heart can result in a similar cardioprotective effect known as remote ischaemic preconditioning (rIPC). Maintenance of Ca²⁺ homeostasis during ischaemia and reperfusion is important both for protection against reperfusion injury (2) and for the re-establishment of function post MI. We have therefore examined how IPC affects Ca²⁺ homeostasis in rat ventricular myocytes from hearts subject to IPC and in control myocytes following rIPC.

Isolated hearts were subject to IPC consisting of 3 cycles of 5 min global ischaemia and reperfusion. Ventricular myocytes were isolated from control and IPC hearts by enzymatic dissociation (3). The first 3ml of perfusate eluted from the heart following each period of preconditioning ischaemia were retained for use in rIPC experiments. IPC was achieved by treating cells with this perfusate for 10 minutes followed by a 5 minute washout period. Measurements of [Ca²⁺] were made using Fura-2, L-type Ca²⁺ current was measured using whole-cell patch clamp. Values are mean±SEM compared by ANOVA following by Tukey’s post-test (n=hearts, cells).

IPC increased systolic [Ca²⁺] from 468±17nM (n=10, 70) in control cells to 547±30nM (n=6, 28) in IPC cells although this was not significant, while rIPC reduced systolic [Ca²⁺], to 413±21nM (n=4, 35, P<0.05). SR Ca²⁺ content measured by application of 20mM caffeine was unaltered following IPC (430±16nM (n=10, 46) in control cells, 386±21nM (n=6, 24) in IPC cells, 409±34nM (n=4, 18) in rIPC cells). The time constant for the decay of the Ca²⁺ transient (τ) was faster in IPC cells (119±4ms, n=6, 27) than in control cells (132±3ms, n=10, 71, P<0.05), suggesting that the rate of Ca²⁺ uptake into the SR was increased. The decay of the Ca²⁺ transient was also faster following rIPC (111±2ms, n=4, 35, P<0.01), mirroring the changes observed for IPC. The time constant for the decay of the caffeine-evoked Ca²⁺ release (τ) was faster in both IPC cells (2.5±0.1s, n=6, 16, P<0.01) and rIPC cells (2.9±0.2s, n=4, 16, P<0.01) than in control cells (3.7±0.3s n=10, 28), indicating

130P
that the activity of NCX is increased by IPC and rIPC. The L-type Ca$^{2+}$ current at 0mV was significantly increased from -9.1±0.7pA/pF (n=2, 10) in control cells to -14.6±1.3pA/pF (n=2, 10) following IPC (P<0.01).

Our data shows that IPC and rIPC modulate pre-ischaemic Ca$^{2+}$ handling in ventricular myocytes and this may be involved in their protective effect. However, significant differences are apparent between myocytes isolated following whole heart IPC and rIPC of naïve myocytes.


HET is funded by the BHF.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC139**

**Computer model of the rat ventricular myocyte with different $I_{Ca}$ inactivation at the peripheral and t-tubule membranes**

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We have previously developed a mathematical model of electrical activity and ion handling in the rat ventricular myocyte (Pásek et al., 2006), which includes a quantitative description of the transverse-axial tubular system (TATS). The model has been used to explore the role of the t-tubules in determining the electrical and mechanical activity of the myocyte. However, recent experimental data have shown that some aspects of trans-sarcolemmal Ca$^{2+}$ flux and intracellular Ca$^{2+}$ dynamics are more complex than formulated in the model, and that the tubular fractions of some trans-sarcolemmal ion fluxes are different from those used previously (Pásek et al., 2008; Chase & Orchard, 2011). We have, therefore, developed the model further, to incorporate a modified description of calcium current ($I_{Ca}$) and Ca$^{2+}$ handling, in particular: (i) separating the original single dyadic space, and single sarcoplasmic reticulum (SR) release compartment, into two, one adjacent to peripheral membrane and the other adjacent to tubular membrane, and incorporating peripheral and tubular intracellular subspaces (Shannon et al., 2004); (ii) reformulating $I_{Ca}$ inactivation to reflect enhanced Ca$^{2+}$-dependent inactivation due to SR Ca$^{2+}$ release at the TATS, compared with the peripheral membrane (Brette et al. 2004); (iii) incorporating a quantitative description of diffusion of exogenous Ca$^{2+}$ buffers (e.g. EGTA, BAPTA) between the pipette and intracellular compartments; (iv) incorporating the most recent experimentally-derived tubular fractions of Ca$^{2+}$ flux pathways (Chase & Orchard, 2011). The modified model is stable for at least 5 hours of simulated activity, with appropriate and reversible changes of ion concentrations with changes of activity. It reproduces the experimentally-observed effect of SR inhibition and exogenous Ca$^{2+}$ buffers on $I_{Ca}$ inactivation at the tubular and peripheral membranes, and has been used to explore the effects of activity-induced changes of ion concentrations in the tubular lumen on excitation-contraction coupling.


Pásek M et al. (2008). Prog Biophys Mol Biol 96, 244-257.


This work has been supported by the project AV0Z 20760514 from the Institute of Thermomechanics of the Czech Academy of Sciences, by the project MSM 0021622402 from the Ministry of Education, Youth and Sports of the Czech Republic, and by the British Heart Foundation.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC140**

**M Cells in Humans: Action Potential Modelling and Impact on APD Distribution in Cardiac Tissue**

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**Introduction:** The human ventricle has been thought to consist of epicardial (epi) and endocardial (endo) cell types. A recent experimental study [1] has confirmed the midmyocardial (M) cell type in the human ventricle, along with transitional midmyocardial (TM) cell type. M cells have markedly prolonged action potentials (APs) and distinct ionic current properties. We hypothesised that human ventricles consist of four cell types, and M along with TM contribute to tissue propagation properties. We developed mathematical models of M and the newly found TM cell types. Experimentally observed conduction patterns using a heterogeneous 1D model of virtual human ventricular tissue were reproduced in simulations.

**Methods:** Experimental data of major ionic currents were incorporated into a recent biophysically detailed model [2] to reproduce human M and TM cell APs. In brief, a late sodium current ($I_{NaL}$) component was added to the sodium current ($I_{Na}$), the inward potassium current ($I_{Ks}$) conductance was reduced by 26%, and the slow rectifier potassium current ($I_{Ks}$) conductance was reduced by 54% to simulate M cell AP. A TM model was similarly constructed. Single cell APs were compared at different pacing cycles for the four different cell types. A 1D virtual tissue strand model incorporating electrophysiological heterogeneity was constructed based on experimental data [1]. An established computational environment, CHASTE [3], was adopted in the simulations. Cell APDs for the four cell types were computed and compared to experimentally observed APDs in various single cells at different pacing rates. The 1D strand model was used to simulate various conduction patterns.

**Results:** The model reproduced the reported prolonged APs and rate adaptation characteristics of M cells (Fig. 1, A). The difference between M and epi repolarization is primarily due to $I_{NaL}$. ApD restitution is steeper in M cells than in the other cell types, indicating the augmented electrical heterogeneity due to M cell type. APD distribution along the 1D strand revealed the role of M cells in augmenting transmural heterogeneity (Fig. 1, B). At slow rates AP is longer in M and endocardial regions than in epicardium. M and TM cells distinctly regulated conduction patterns in the 1D strand model.
Conclusions: $I_{\text{APD}}$ was found to be the major contributor to the prolonged M cell AP. Our 1D strand model of ventricular tissue successfully reproduces the profile of APD distribution across the ventricular wall. It is a useful tool for investigating the role of M and TM cells in ventricular conduction patterns.

We found that the % viability following ischaemia/reperfusion of control myocytes was $61.1 \pm 2.7\%$ (n=35,20), which was significantly increased in rIPPC to $71.6 \pm 2.7\%$ (n=28,20; p<0.05) and rIPostC $78.4 \pm 2.7\%$ (n=31,20; p=0.001). We used this model to look at the effect of non-specific Opioid (Naloxone) and Adenosine (8-p-Sulphophenyl Theophylline, 8p-SPT) receptor inhibitors, with the inhibitor present throughout the experimental protocol. Our data show that 100μM Naloxone reversed the protective effects of rIPC, reducing viability to $60.1 \pm 5.95\%$ (n=11, 7; p>0.05) and rIPostC to $63.7 \pm 3.6\%$ (n=12, 7; p<0.05). We also found that 100μM 8p-SPT reversed the protective effects of rIPC reducing viability to $55.8 \pm 6.3\%$ (n=9, 8; p<0.05) and rIPostC to $67.1 \pm 5.3\%$ (n=11, 8; p>0.05). Our data shows that rIPC and rIPostC protect against reperfusion injury at the cellular level. The data also shows that Opioids and Adenosine are likely triggering molecules of rIPC and rIPostC.

This research was supported by the BHF. GRH is an intercalated BSc student.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Impact of age on structural remodelling in the spontaneously diabetic Goto Kakizaki rat

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The cardiac effects of type 2 diabetes mellitus (T2DM) are known to be most pronounced in the elderly. Chronic hyperglycaemia (HG) may underlie this process by producing structural changes that alter left ventricle (LV) function. However, the mechanisms via which HG affects the heart specifically to accelerate the effects proper to ageing are relatively unknown. This study investigated the effect of ageing on dysglycaemia-related myocardial remodelling and associated molecular events in the spontaneously diabetic Goto Kakizaki (GK) rat. Mildly diabetic GK rats 2 months (young) and 18 months (old) of age and age-matched Wistar controls were humanely killed in accordance with Institutional Regulations after administration of an oral glucose tolerance test and measurement of blood glucose collected from the tail vein to confirm HG. LV samples were collected for histological, immunohistochemical and gene expression analyses using methods described elsewhere [1].

The results (mean±SEM) showed that interstitial fibrosis area fraction (1.44±1.02% in young and 7.38±0.50% in old GK rats vs. 1.00±0.13% and 5.87±0.54% in respective age-matched controls, Student’s t-test; p<0.05), mean heart weight/weight ratio (0.32±0.59 ratio units in young and 1.41±0.16 ratio units in old GK rats vs. 0.23±0.48 and 1.22±0.2 ratio units in respective controls, p<0.05), LV wall thickness (3.15±0.46 mm in young and 3.35±0.17 mm in old GK rats vs. 2.98±2.07 and 3.08±2.39 mm in respective age-matched controls, p<0.05) and myocyte diameter (9.93±2.69 μm in young and 11.34±0.54 μm in old G-K rats vs. 9.11±2.55 and 9.43±0.35 μm in respective age-matched controls, p<0.05) demonstrated a trend towards greater fibrosis proliferation and LV hypertrophy with ageing. The effect was also accentuated by the presence of T2DM in the GK rats (2-way ANOVA, p<0.05). These alterations were concomitant with up-regulated active profibrotic Transforming growth factor beta 1 (TGFβ1) activity (2.89±0.60 vs. 5.47±1.48 pg/mg (total protein) in young control and GK rats and 2.12±0.54 vs. 5.4±0.74 pg/mg (total protein) in old control and GK LV, p<0.05) and gene expression in the diabetic groups relative to control. (1.15±3.32 vs. 1.76±22 ratio units normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in young control vs. GK rats and 0.97±0.14 vs 1.92±0.17 ratio units normalised to GAPDH in old control vs G-K rats, p<0.01). Finally, levels of phosphorylation of pro-hypertrophic Akt and p70S6K were also significantly up-regulated in dysglycaemic groups (p<0.05), but age related effects were not apparent (2-way ANOVA, p<0.05). The results of this study have demonstrated that chronic moderate elevations in HG can aggravate age-induced remodelling changes in the GK myocardium. In addition, elevated levels of TGFβ1 and altered Akt signalling may be key intermediaries in this process.


**Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.**

**PC143**

**Carbonic anhydrase activity in rat cardiac myocytes is extra-mitochondrial**

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The carbonic anhydrases (CA) catalyse the hydration of CO\(_2\) to HCO\(_3^-\) and H\(^+\) ions, and the reverse reaction. Their activity has been shown to facilitate fluxes of H\(^+\) ions, HCO\(_3^-\) ions and CO\(_2\) across membranes and optimise the pH buffering capacity of CO\(_2/\)HCO\(_3^-\) buffer. Protein expression studies on ventricular myocytes have demonstrated immuno-reactivity for CAIV, IX and XIV in the sarcoleplasmic reticulum, CAIV and XIV at the sarcolemma (Schiebe et al, 2006) and CA in mitochondria (Vaananen et al 1991). Functional studies have demonstrated modest intracellular CA activity in guinea-pig myocytes (Leem & Vaughan-Jones, 1998). Mitochondria are a principal source of CO\(_2\), a CA substrate. Previous work on mitochondrial suspensions using radio-tracers has found no CA activity (Dodgson et al, 1980). In the present work, we have investigated the distribution of CA activity in rat ventricular myocytes using pH-fluorophores. Carboxy-SNARF-1 was AM-loaded into intact myocytes. By varying the loading time (5 min to 2 hrs), it was possible to manipulate the degree of dye partitioning between the intra- and extra-mitochondrial compartments (higher mitochondrial signal with longer loading). The fraction of dye held in mitochondria was estimated at the end of each experiment by releasing cytosolic dye with 0.005% saponin. CA activity was measured by raising CO\(_2\) in cells by switching the extracellular solution from CO\(_2\)-free (Hepes-
buffered at pH 7.4) to 5% CO2 (also containing 22 mM HCO3- to bring pH to 7.4). This drives intracellular CO2 hydration, lowering the pH reported by carboxy-SNARF-1 at a rate proportional to CA activity. For purely cytosolic dye loading, the CO2 hydration rate was 0.64±0.12(SEM) s^-1 in controls and 0.22±0.03 s^-1 in the presence of the CA inhibitor acetazolamide (ATZ; 100 μM), giving a CA activity of 2.85. A negative correlation (r=-0.23; P=0.032 for one-tailed test; n=30) was observed between CA activity and the compartmentalisation of dye in mitochondria. This result suggests that mitochondria have lower CA activity than cytosol. Further experiments were performed on isolated mitochondria (isolation according to Das et al, 2003). Mitochondria were AM-loaded with BCECF and re-suspended in 140 mM K+ solution at pH 7.2. Upon addition of CO2-saturated buffer, the mitochondrial matrix acidified. The rate of acidification was not affected by ATZ (0.017±0.0021 vs 0.017±0.0007 pH s^-1; n=6), suggesting negligible CA activity. Such intracellular CA activity distribution (with a predominantly extra-mitochondrial locus) would favour extra-mitochondrial CA hydration rate was 0.64±0.12(SEM) s^-1 in controls and 0.22±0.03 s^-1 in the presence of the CA inhibitor acetazolamide (ATZ; 100 μM), giving a CA activity of 2.85. A negative correlation (r=-0.23; P=0.032 for one-tailed test; n=30) was observed between CA activity and the compartmentalisation of dye in mitochondria. This result suggests that mitochondria have lower CA activity than cytosol. Further experiments were performed on isolated mitochondria (isolation according to Das et al, 2003). Mitochondria were AM-loaded with BCECF and re-suspended in 140 mM K+ solution at pH 7.2. Upon addition of CO2-saturated buffer, the mitochondrial matrix acidified. The rate of acidification was not affected by ATZ (0.017±0.0021 vs 0.017±0.0007 pH s^-1; n=6), suggesting negligible CA activity. Such intracellular CA activity distribution (with a predominantly extra-mitochondrial locus) would favour extra-mitochondrial CA hydration and may accelerate CO2 venting from the mitochondrial matrix. This would reduce matrix acid-loading and hence the degree of respiratory end-product inhibition.


Supported by the Medical Research Council, Royal Society and British Heart Foundation. We thank Dr Robert Wilkins for granting access to the spectrofluorometer.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC145

Changes of SERCA activity have proportionately smaller effects on sarcoplasmic reticulum calcium content

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The sarcoplasmic reticulum (SR) provides the majority of calcium to the systolic calcium transient. It is this rise in intracellular calcium that causes cardiac systole. The SR is, therefore, important for maintaining cardiac contractility. SR calcium content is replenished by the sacro(endo)plasmic reticulum calcium ATP-ase (SERCA). It might be expected that reducing SERCA activity would result in an equally decreased SR calcium content, impairing contractility. However, Andersson et al. (2009) have shown, in a SERCA2 conditional knock-out mouse model, that reduction of cardiac SERCA2 expression to 5% of control levels only decreased SR calcium to 38% of control. We now investigate how acute changes to SERCA activity (k_SERCA) affect SR calcium content in the normal ventricular myocyte. Single isolated ventricular myocytes from rats were used and intracellular calcium concentration measured with fluo-5. Experiments were performed at 37 °C, using the perforated patch voltage-clamp technique. Systolic calcium transients were evoked with depolarizing pulses at 0.25 Hz. k_SERCA was quantified from the rate constant of decay of the calcium transient. SR calcium content was determined from the integral of the caffeine-evoked Na-Ca exchange current. 1 μM thapsigargin was applied to the cell to slow k_SERCA to varying levels. The data reported is expressed as the mean ± SEM. Significance was tested using One Way Repeated Measures ANOVA. Upon repeated application of thapsigargin k_SERCA slowed by 65% (Figure 1) from 7.4 ± 0.8 s^-1 to a final value of 2.6 ± 1.5 s^-1 (n = 4 – 13 cells, p = 0.001) and SR calcium content decreased by 46% from 82 ± 5 μM to 44 ± 5 μM (n = 4 – 13 cells, p < 0.001). Using this data we found that SR calcium content was proportional to (SERCA activity)^1/m where the mean value for m was 2.59. Therefore, a large reduction in k_SERCA results in a proportionately smaller decrease in SR calcium content (Figure 2). This relationship can be accounted for by the fact that calcium efflux from the SR is a steep function of SR calcium content. A decrease in k_SERCA will decrease SR calcium content and even a small reduction of SR calcium will lower calcium release to balance uptake.

Figure 1: Normalised systolic calcium transients in the same cell after repeated application of 1 μM thapsigargin. This shows the rate of decay of the calcium transient slows with each successive thapsigargin treatment.

Figure 2: The effects of changes of SERCA activity on SR calcium content. Data was calculated by measuring k_SERCA and SR calcium content following exposure to thapsigargin. Measurements are normalised to control values in each cell. The dashed line is the line of identity. The solid line is a fit to SR Ca content = 100*(k_SERCA/100)^m where m = 0.38.


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Carbonylation contributes to RyR2 dysregulation and dysynchronous sarcoplasmic reticulum Ca\(^{2+}\) release in a rat model of type 1 diabetes mellitus

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The force of contraction of the heart is reduced during type 1 diabetes mellitus (T1DM) [1]. Studies attribute this defect in part to dysynchronous Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) arising from variations in activities of type 2 ryanodine receptors (RyR2). To date, mechanism(s) underlying RyR2 dysregulation during T1DM remain poorly defined. The streptozotocin-induced (45-50 mg/kg; ip, n>40) rat model of T1DM in combination with echocardiography, ex vivo haemodynamics, video detection, electron and confocal microscopy, Western blots, ligand binding and lipid bilayer assays, mass spectrometry and site-directed mutagenesis were used to evaluate whether changes in dyad junction architecture and/or carbonylation are contributing factors [1]. The study had the relevant ethical clearances from the Ethics Committees of the collaborating Universities.

The results show significant reduction (Student’s t-test; p<0.05) and abnormal ventricular and myocyte contractions after 8 weeks of T1DM. Typically, mean cardiac fractional shortening was reduced by 25.2 ± 3.2% and extent of myocyte shortening by 30.3 ± 6.7%, respectively. Diabetic myocytes showed increased 4.4-fold spontaneous Ca\(^{2+}\) and dysysncronous-evoked Ca\(^{2+}\) releases from the SR. Electron microscopic analyses revealed no significant disruption in dyad junction architecture. RyR2 protein remained unchanged although total [3H]-ryanodine binding was reduced by 45.6 ± 4.2% (t-test; p<0.05). Trypsin digestion and mass spectrometry revealed carbonyl adducts on R1611, K2190, R4462 and R4683 of RyR2. Mutating impacted residues singly and in combination to glycine, tyrosines or tryptophans to mimic charge neutralization and increase in bulk induced by carbonylation afforded two distinct channel phenotypes in lipid bilayer assays. The results show channels with 2 to 9 fold increases in responsiveness to low cis (cytoplasmic) Ca\(^{2+}\) and channels with >10-fold reduction in responsiveness to low cis Ca\(^{2+}\). Insulin-treatment minimized adduct formation, RyR2 dys-regulation and cardiac function loss.

In conclusion, the results show that dysynchronous-evoked Ca\(^{2+}\) release from SR during T1DM stems in part from carbonylation-induced alterations in responsiveness of RyR2 to influxed Ca\(^{2+}\), thus, providing new mechanistic insights into the pathogenesis of diabetic cardiomyopathy.


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Concurrent Optical Mapping of Voltage and Calcium in Rat Isolated Hearts using One Camera

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Concurrent measurement of membrane voltage (Vm) and intracellular calcium ([Ca\(^{2+}\)]\(_i\)), key parameters underlying excitation-contraction coupling, is necessary for exploring the cross-talk between these two processes. The development of voltage- and ion-sensitive fluorescent dyes has enabled non-contact measurements, with increasing spatial and temporal resolution. The use of dual camera systems is the most commonly used approach to visualise Vm and [Ca\(^{2+}\)]\(_i\). However, this approach is technically challenging and expensive. We have developed a single-camera system utilising frame-accurate light-emitting-diode (LED) activation to measure Vm and [Ca\(^{2+}\)]\(_i\) ratiometrically, using Di-4-ANBDQPO (for Vm) and Fura-2 (for [Ca\(^{2+}\)]\(_i\)). High-speed coordination between the LEDs (ranging from UV to red) and a 128x128 EMCCD camera system (Cascade; Photometrics) is achieved via custom, micro-processor based control, which also allows high flexibility in LED excitation sequence. Emission fluorescence is passed through a multiband filter.

As a proof-of-principle application, ratiometric Vm and [Ca\(^{2+}\)]\(_i\) signals were measured during electrical and mechanical stimulation at the same location on the left ventricle in rat hearts. Hearts (n=8) were excised after cervical dislocation (female SD, 10-12wk), according to Sch1 of the Home Office Animals (Scientific Procedures) Act of 1986, and Langendorff perfused with Krebs solution (in mM: NaCl 123, CaCl\(_2\) 1.8, KC\(_2\) 4, MgCl\(_2\) 1.2, Na\(_2\)HPO\(_4\) 1.4, NaHCO\(_3\) 24, Glucose 10). Fura-2 (10μM) was reperfused for 40 min after initial bolus injection, and Di-4-ANBDQPO was applied by bolus injection (20μl of 27.3mM stock in EtOH, over 5 min to 25 ml of perfusate). The speed of action potential propagation and the time delay between the peaks of Vm and [Ca\(^{2+}\)]\(_i\) were compared for the two modes of stimulation.

No significant difference were found in propagation speed of electrically and mechanically triggered excitations (0.65±0.04mm/ms vs 0.65±0.07mm/ms, respectively). Furthermore, there was no significant difference in the time-delay between peak Vm and [Ca\(^{2+}\)]\(_i\), ([22.0±1.4ms vs 22.5±1.3ms for electrical and mechanical stimulation, respectively.

We present a simple, versatile and scalable technique for concurrent ratiometric imaging of Vm and [Ca\(^{2+}\)]\(_i\), in the isolated rat heart. For proof-of-principle, we integrated this technology with ECG-timed local electrical or mechanical stimulation to study mechano-electric coupling at the organ level, and show that activation patterns, down-stream of the ectopic excitation, are not dissimilar regardless of the trigger.

This work was supported by the BBSRC, and the BHF.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Acute caloric restriction reduces free radical damage in heart, reperfusion arrhythmias and myocardial infarction size in rats


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Reduced coronary perfusion in acute coronary syndrome causes myocardial ischemia. However, therapeutic process of restoring blood flow to the ischemic myocardium, i.e. reperfusion therapy, can induce injury. In the post-ischemic heart reactive oxygen species (ROS) formation is increased and could serve as a mechanism of this myocardial reperfusion injury (1). ROS formation and subsequent oxidative damage is reduced by, long-term (months) and short-term (weeks), caloric restriction (2,3) and is probably modulated by the cell redox state (4). The effect of acute caloric restriction (couple days immediately before the ischemic episode) has not yet been properly examined.

We investigated whether the 3-day fasting affects production of ROS, incidence of ventricular arrhythmias during ischemia/reperfusion, myocardial infarction size and redox state of mitochondria.

Adult male Wistar rats, anesthetized by sodium pentobarbitone (60 mg/kg body weight, intraperitoneally) were used. We analyzed production of the lipid peroxidation end products (lipofuscin-like pigments, LFP) in the heart tissue according to Goldstein & McDonagh (5). LFP concentration was expressed in relative fluorescence units (RFU) per mg of the tissue weight. Open-chest rats were intubated through the tracheotomy with a cannula connected to a rodent ventilator (Ugo Basile, Italy) and ventilated with room air at 65-70 breaths/min (tidal volume of 1.2 ml/100 g body weight). Then, the animals were subjected to 20-min left anterior descending coronary artery occlusion followed by reperfusion. A single lead ECG was recorded and arrhythmias were assessed. The infarction size in excised hearts was determined by triphenyltetrazolium chloride staining and it was normalized to area at risk (IS/AR). To assess redox state of the mitochondrial compartment we analyzed, in the excised hearts, acetocetate/β-hydroxybutyrate ratio (AA/BHB) correlating with NADH/NAD+ ratio.

All results are expressed as means ± S.E.M. The statistical significance of differences was determined by one-way ANOVA and subsequent Fisher's PLSD test and the Games/Howell post hoc test, as appropriate.

Three day fasting reduced LFP production in heart tissue: 1.9±0.1 RFU in fasting rats (n=9) vs. 3.1±0.3 RFU in controls (n=7), p<0.005. It did not affect ischemic arrhythmias. During early reperfusion the number of premature ventricular complexes was reduced in fasting rats (n=12, 12.5±5.8) compared to controls (n=11, 194.9±21.9), p<0.005, as well as ventricular tachycardia duration (0.6±0.4 sec vs. 18.8±2.5 sec), p<0.005. In fasting rats (n=6) the IS/AR reached 48.5±3.3 % while in controls (n=5) it was 74.3±2.2 %, p<0.005. AA/BHB ratio in fasting rats (n=11) was 0.03±0.01, while in controls (n=10) it was 0.17±0.04, p<0.05.

Acute caloric restriction limits free radical damage in heart, reperfusion ventricular arrhythmias and myocardial infarction size in rats. This protective effect could be induced by changes of mitochondrial redox state.


Supported by the Grant Agency of Charles University (60009/2009) and Cardiovascular Research Centre (MSMT 1M 0510).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Changes of action potential duration and intracellular calcium following a change of pacing frequency in sheep ventricular myocytes

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Action potential duration (APD) decreases following an increase in heart rate and vice versa. Changes in APD can occur with a time course of hours to days, a phenomenon known as cardiac memory. In the short term however, changes occur both rapidly (within a second) and more slowly, with a time course in the order of tens to a few hundreds of seconds. The rate constant of APD change is typically faster when rate is increased compared to when it decreases. APD changes over these time courses can also be seen in isolated ventricular myocytes. Our aim was to determine whether the slow changes in APD observed upon alteration of pacing frequency would correlate with those of intracellular calcium ([Ca2+]i).

Young (~18 months) Sheep were killed in accordance with The Home Office Animal (Scientific Procedures) Act 1986 for enzymatic isolation of left ventricular mid myocardial myocytes. Myocytes loaded with the calcium indicator Fura-2 were current-clamped via perforated patch and paced at 0.25 Hz, 1 Hz and 0.25 Hz sequentially, to steady state Fura and Fura ratio values. APD was taken as the duration at 90 % repolarisation (APD90).

When pacing frequency was increased from 0.25 Hz to 1 Hz, the rate constant of change of both APD90 and peak systolic [Ca2+]i were faster than when frequency was decreased. However the changes of [Ca2+]i were generally faster than those of APD90. For example, on increasing pacing frequency to 1 Hz, the change of peak systolic [Ca2+]i was approximately 5 fold faster than that of APD90 (APD, 0.056 ± 0.006 s-1; peak [Ca2+]i, 0.24 ± 0.05 s-1, n = 13, p<0.05). When pacing frequency was subsequently decreased to 0.25 Hz, the change in peak systolic [Ca2+]i was still faster than that of APD90 (APD, 0.029 ± 0.006 s-1; peak [Ca2+]i, 0.13 ± 0.04 s-1, n = 9, p=0.07). Diastolic [Ca2+]i changed approximately 10 fold faster than APD90 when pacing frequency was increased from 0.25 Hz to 1 Hz (APD, 0.056 ± 0.006 s-1; diastolic [Ca2+]i, 0.51 ± 0.09 s-1, n = 13, p<0.05). Upon a subsequent decrease of pacing frequency...
to 0.25 Hz, the rate constant of diastolic $[Ca^{2+}]$, change was approximately 15 fold faster than that of APD$_{50}$ (APD$_{0.029} \pm 0.006 s^{-1}$; diastolic $[Ca^{2+}]$, 0.45 $\pm$ 0.06 s$^{-1}$, n = 9, p<0.05). The hysteresis of APD$_{50}$ and peak and diastolic calcium (which both represent an absolute $[Ca^{2+}]$ in the cytoplasm), suggests that the slow change of APD$_{90}$ is not dependent on a change of $[Ca^{2+}]$.

This work was supported by The British Heart Foundation. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Measurement of myocardial sarcoplasmic resistivity by dielectrophoresis and impedance analysis

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Action potential propagation velocity depends on the intracellular resistance, which in myocardium is the sum of gap junction resistance and the resistivity of the sarcoplasm. The value of sarcoplasmic resistivity has not been routinely measured in mammalian myocardium and thus its overall contribution to intracellular resistance is unclear. We compared two different approaches, dielectrophoresis (the induced motion of particles in non-uniform electric fields) and measurement of cellular impedance to measure sarcoplasmic resistivity, Rs. Experiments used isolated atrial myocytes and strips from guinea pigs, and an immortalised atrial cell line, HL-1 (1). HL-1 cells and freshly isolated atrial myocytes were used for dielectrophoresis and impedance measurements respectively and were suspended in an isotonic solution of Krebs/sucrose to achieve a solution resistivity of about 350 $\Omega$.cm. The analyses to extract cellular electrical constants have been described (2.3). Atrial strips, maintained in an oil-gap chamber, were also used to measure Rs by passing alternating currents along the intracellular pathway (4). Experiments were performed at 25°C and data are means $\pm$ SD.

Dielectrophoresis yielded a value for Rs of 126$\pm$11 $\Omega$.cm, which increased to 271$\pm$49 $\Omega$.cm when cells were suspended in sucrose solution omitting divalent cations. Impedance measurements on isolated cell suspensions offered two methods to estimate Rs, from the limiting impedance at high ($>$60 kHz) and from the frequency at which maximum cellular admittance (1/impedance) was measured: values of 146$\pm$42 and 136$\pm$59 $\Omega$.cm were obtained respectively. Finally, the impedance of the intracellular pathway was measured and the limiting value at high frequencies ($>$200 kHz) used, a value of 145$\pm$37 $\Omega$.cm was obtained.

The values of sarcoplasmic resistivity as measured by four independent physical methods yielded very consistent values, with a mean of 138 $\Omega$.cm, representing a value about 2.5-times that of isomolar Tyrode’s solution. In atrial tissue this value represents about one-half of the total intracellular resistance (287$\pm$94 $\Omega$.cm, unpublished data S Salvage and RI Jabr), the remainder due to gap junction resistance. Thus, Rs itself will significantly influence the value of action potential conduction velocity and as a result substantial changes to gap junction resistance would have to occur before it altered conduction velocity.


Poster Communications

PC150

Spontaneous differentiation of embryonic stem cells leads to line-dependent functional diversity of cardiac myocytes

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Introduction: Pluripotent stem cell-derived cardiomyocytes are thought to be an in vitro model for the early heart development and in drug testing and hold great potential for cellular cardiomyoplasty. They have been shown to resemble different subtypes of cardiomyocytes in regard of their electrophysiological properties. So far, it is not clear to which extent different embryonic stem cell lines are comparable in their quality of differentiation into cardiomyocytes.

Methods: Two different transgenic stem cell lines (CGR8/AMP1GX-7, Doss 2007) and D3/apig44, Kolossov 2005) were grown on feeder cells in the presence of leukaemia inhibitory factor and differentiated by the hanging drop method. In order to obtain alpha-myosin heavy chain positive cardiomyocytes at a high degree of purity, puromycin was added. After 14 days of differentiation, transmembrane potentials were recorded with microelectrodes at 37°C.

Results: Without purification, action potential frequency in D3/apig44 (n=45) was markedly higher than in CGR8/AMP1GX-7 (n=20) (7.7$\pm$0.1 Hz vs. 1.7$\pm$0.2 Hz, p<0.001). The action potential density of D3/apig44 was markedly shorter (APD20: 7.0$\pm$1.1 Hz vs. 36.5$\pm$2.2 Hz, APD50: 14.6$\pm$0.2 Hz vs. 80.6$\pm$10.2 Hz, APD90: 29$\pm$0.4 Hz vs 188.9$\pm$13.2 Hz, all p<0.001). Comparison of purified (n=28) and non-purified (n=45) D3/apig44 showed a slightly smaller frequency of purified ESC-CM clusters (7.2$\pm$0.1 Hz vs. 7.7$\pm$0.1 Hz, p<0.01) and an increase in APD20 (7.0$\pm$0.2 Hz vs. 8.4$\pm$0.4 Hz, p<0.05. and in APD90 (20$\pm$0.4 Hz vs. 29.6$\pm$0.4 Hz, p<0.05). In contrast, purified CGR8/AMP1GX-7 (n=20) had a strong decrease of APDs with purification compared to unpurified CGR8/AMP1GX-7 (APD20: 26.3$\pm$3.1 Hz vs. 36$\pm$5 Hz, APD50: 50.1$\pm$5.1 Hz vs. 80.6$\pm$10.2 Hz, APD90: 79.8$\pm$7.3 Hz vs. 188.9$\pm$13.2 Hz, all p<0.05). The frequency did not significantly change.

Conclusions: The spontaneous differentiation of D3/apig44 and CGR8/AMP1GX-7 leads to electrophysiologically distinct cardiomyocyte phenotypes in regard of beating frequency, APD and effect of purification under control of the alpha-myosin heavy chain-promoter. CGR8/AMP1GX-7 strongly resemble the ventricular phenotype, while D3/apig44 share many properties with sino-atrial cells. This questions the comparability and validity of the spontaneous differentiation of embryonic stem cells as a model for cardiac development and drug
testing and underlines the necessity of unraveling factors to control cardiac subtype differentiation.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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PC152

Caveolae Limit β2 Adrenoceptor-derived cAMP Signals in the Adult Cardiac Myocyte


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Stimulation of β1-adrenoceptors (ARs) produces marked positive inotropic and lusitropic responses in the cardiac myocyte, which are absent following β2-AR stimulation. Compartmentation of β2-AR-derived cAMP-PKA signals is thought to contribute to this functional discrepancy. We are interested in how caveolae (sarcosomal invaginations rich in cholesterol, sphingolipids and caveolin-3) contribute to this compartmentation in the adult rat ventricular myocyte (ARVM). We have previously observed substantial augmentation of β2-AR-mediated inotropic and lusitropic responses following cholesterol depletion of ARVM (1). Here, we (i) confirm the specificity of this effect on caveolae by multiple approaches and (ii) investigate which proteins could play a role via residence in caveolae. ARVM were treated with either 1 mM methyl-β-cyclodextrin (MBCD) to deplete cholesterol, or conjugated MBCD:cholesterol (molar ratio 1:8). In separate experiments, some cells were incubated with 0.5 μM TAT-35SD (a cell-permeable peptide inhibitor of caveolin-3 scaffolding interactions) or TAT-Scram (scrambled sequence). β2-AR stimulation was achieved with 10 μM zinterol in the presence of 300 nM CGP20712A; cell shortening was measured with edge-detection software. Protein and phospho-protein levels were determined by immunoblotting. ARVM were fractionated on a discontinuous sucrose gradient. Statistical significance was assessed with the Student’s t-test.

Following β2-AR stimulation, both shortening and time to half relaxation were enhanced in MBCD-treated compared to control cells (70.2 ± 9.7% vs 49 ± 5.8% and -13.3 ± 1.3% vs -5.4 ± 1.3% respectively; P<0.001, n=13-20). These inotropic and lusitropic effects of MBCD were greatly attenuated by 87 and 77% respectively when MBCD was conjugated to cholesterol prior to treatment of cells (P<0.01, n=11-13). We have previously linked MBCD effects to a selective increase in protein kinase A (PKA) phosphorylation of phospholamban at Ser16 (pPLB), but not troponin I at Ser23/24 (pTnI) (1). Pre-incubation of ARVM with TAT-35SD peptide also enhanced pPLB during β2-AR stimulation compared to that with TAT-Scram (46.1 ± 6.3-fold vs 2.2 ± 0.8-fold respectively; P<0.01, n=3). Levels of pTnI were unaltered by the same treatments (P>0.05, n=3).

Finally, we detected caveolin-3, β2-AR, Gαi3, adenylyl cyclase 5/6, PKA RII, protein phosphatase 2a, and G-protein-coupled receptor kinase, but not phosphodiesterase 3A or 4D, in caveolar raft fractions.

We conclude that in ARVM (i) the effects of MBCD on β2-AR stimulation are specific to cholesterol depletion and caveolae disruption and (ii) the signal components resident in caveolae have the potential to compartmentalise cAMP by facilitating processes that limit the magnitude and propagation of cAMP signals, including β2-AR phosphorylation and β2-AR – Gi coupling.


Supported by the Medical Research Council.

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PC153

Ageing produces a distinct molecular fingerprint in the failing ovine ventricular myocardium

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Heart failure is characterised by decreased contractile performance of the heart. Dibb et al (2004) have previously demonstrated, in an ovine model of ageing, differences in intracellular calcium homeostasis in the ventricle are similar to those in early heart disease (Mørk et al. 2007). The aim of the present work was to extend these observations and determine if heart failure in the aged results in different response of calcium homeostatic proteins to that in the young. Under isoflurane anaesthesia (2–4% in oxygen) sheep were instrumented with a pacemaker and pacing lead. Post-operative analgesia (meloxicam 0.5mg/kg) and antibiotics (enrofloxacine 2.5mg/kg) were provided for 24 hr. Heart failure was induced by rapid ventricular pacing (Briston et al., 2011) for 4–5 weeks. Following pentobarbital euthanasia (200 mg/kg iv) samples of left ventricular myocardium were snap frozen. Age-matched non-instrumented animals served as controls. Western blotting was performed using standard methods and protein immunoreactivity quantified by chemiluminescence.

Table 1 summarises the percentage changes in expression for several candidate proteins known to influence intracellular calcium homeostasis. Ageing alone resulted in a decrease in the calcium buffer calsequestrin and increases in protein phosphatase 1 and 2a (P<0.05, Students t-test). Qualitative differences were observed in the protein expression profiles between young and aged failing hearts (relative to age-matched control tissue). Notably, in comparison to young failing hearts where no change in SERCA or the SERCA : phospholamban ratio or CAMKIIδ was observed, in aged-failing hearts these were decreased. Conversely, in young failing hearts protein phosphatase expression increases whereas in aged failing myocardium neither PP1 nor PP2a changed. In summary, there appears to be distinct calcium homeostatic protein expression profile differences between young and aged failing hearts. Whether these differences result in alterations to the properties of the Ca2+ transient remain to be determined.
Table 1. Summary of changes in protein expression in ageing and heart failure

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aged vs Young</th>
<th>Young Heart Failure</th>
<th>Aged Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA2A/PLN ratio</td>
<td>++</td>
<td>++</td>
<td>BC2530</td>
</tr>
<tr>
<td>Pheophytin2</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SERCA2A/PLN ratio</td>
<td>++</td>
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<td>+</td>
</tr>
<tr>
<td>Cadaverine1</td>
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<tr>
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<tr>
<td>Phenol Tt 17 PLN</td>
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<td>CAMEI</td>
<td>++</td>
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</tbody>
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| ++, no change; * P < 0.05 aged control vs young control; $ P < 0.05 vs age-matched control


Supported by the British Heart Foundation and EU 6th Framework (Normacor).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC154

Functional roles of neuronal nitric oxide synthase in NADPH oxidase activity and contraction in angiotensin II-treated rat ventricular myocytes

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Angiotensin II (Ang II) activation of NADPH oxidase is crucial to myocardial pathogenesis. It has been demonstrated that the expression and activities of cardiac nitric oxide synthases (NOS) are up-regulated downstream of Ang II stimulation. However, the functional role of nNOS in left ventricular (LV) myocyte of Ang II remains underdetermined.

Our results show that Ang II (1 μM) significantly prolonged LV myocyte relaxation at 30 min (TR50, field stimulation at 2 Hz, 360°C, P<0.001, between Ang II vs. controls, n=141 and n=201, respectively) whereas such an effect was absent at 1hr or at 2hrs (P=0.6 or P=0.6 comparing to that in controls, n=38 & 41). Surprisingly, TR50 became facilitated after 3hrs incubation with Ang II (P=0.001, n=88) and maintained at this level up to 6hrs (P=0.001 between Ang II 6hrs vs. control, n=49 for Ang II). Similar faster LV myocyte relaxation was also observed with lower doses of Ang II comparing to those of control (3hrs, Ang II 10 mM: P=0.03, n=52; Ang II 100 mM: P=0.001, n=40 or between LV myocytes isolated from rats treated with Ang II via mini-osmotic pump (4 weeks) and those from sham-operated groups (P<0.0001, n=97 vs. 99). The effect was mediated by ATR1, NADPH oxidase and ATR2. Sarcomere shortening was unaffected. Concomitant to time-dependent biphasic changes in TR50 by Ang II, intracellular reactive oxygen species (ROS, 2',7'-dichlorodihydrofluorescein diacetate, H2DCFDA, 10 μM) was increased at 30 min (P=0.02, Ang II vs. control, n=7 and n=10) but decreased to control level after 3hrs (P=0.09, Ang II vs. control, n=33 and n=35). N(omega)-nitro-l-arginine methyl ester (L-NAME, 1 mM) or nNOS inhibitor, S-methyl-L-thiocitrulline (SMTC, 100 mM) elevated intracellular ROS above the control level in the presence of Ang II (P<0.0001 between LV myocytes in Ang II+L-NAME vs. that with L-NAME only, n=29 and n=22; P=0.0001 between LV myocytes in Ang II+SMTC vs. that with SMTC only, n=22 and n=27) and abolished Ang II-facilitation of LV myocyte relaxation (P=0.08, between SMTC only and Ang II + SMTC, n=57 and n=90), suggesting a role for nNOS. Indeed, real time RT-PCR showed nearly two-fold increase of nNOS mRNA. A reducing agent, DTT (1 mM, but not peroxynitrite decomposition catalyst, FePPTS, 10 mM or inhibitors of PKA and CaM kinase II) prevented Ang II-induced faster relaxation (P=0.5 between DTT and DTT+Ang II, n=47 and n=52).

The results clearly demonstrate that myocardial nNOS-derived NO plays an important role in attenuating NADPH oxide-derived ROS to facilitate myocardial relaxation downstream of Ang II through a mechanism involves s-nitrosylation.

This work is funded by National Research Foundation of Korea.

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PC155

Pathological Transmural Electrophysiological Heterogeneity Investigated by Dual Wavelength Optical Mapping

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Introduction: The depth of myocardium in which voltage-sensitive dyes for optical mapping are excited depends on excitation wavelength, due to differences in tissue penetration. Combined short and long wavelength excitation has been used for imaging transmural electrophysiological properties in intact hearts (Walton et al., 2010). We hypothesized that dual wavelength excitation can be used to reveal pathological transmural electrophysiological heterogeneity, such as occurs with ischaemia.

Methods: Langendorff-perfused rabbit hearts (1 kg female, n = 5) were stained with voltage-sensitive dye (20 μM bolus of di-4-ANBDQPO), excitation-contraction uncoupled (10 μM blebbistatin), paced at the apex, and subjected to global no-flow ischaemia. Fluorescence was excited using camera frame-synchronized LEDs (Lee et al., 2011), alternating 470 ± 10 nm (shallow penetration) and 640 ± 10 nm (deep penetration), and >690 nm emission acquired at 922 Hz (64 x 64 pixel, 16-bit CCD camera). Measurements were made for the first 10 min of ischaemia with pacing at 2 Hz, followed by a progressive increase in pacing rate. Computer simulations, representing both electrophysiological changes during no-flow ischaemia (including epicardial border zone) (Tice et al., 2007) and photon scattering effects (Bishop et al., 2006), were used for experimental data interpretation.

Results: Control recordings showed no discernable difference in action potential (AP) morphology between the two excitation wavelengths. However, after 5 min of ischaemia, clear differences appeared, with reduced upstroke velocity and AP
duration at 640 nm compared to 470 nm, which increased with the time of ischaemia. Simulations showed a similar effect and revealed that differences are caused by enhanced wavefront curvature due to increased transmural heterogeneity with ischaemia. When pacing rate was increased, further areas of transmural heterogeneities appeared, resulting in the induction of ventricular tachycardia, which was not the case for control.

**Conclusion:** Thus, dual wavelength optical mapping can reveal pathological transmural electrophysiological heterogeneity in the whole heart and may be useful for investigating its role in the initiation and sustenance of arrhythmias.


1A. Europace 9, 47-58.


TAQ holds a Postdoctoral Fellowship from the Engineering and Physical Science Research Council (EP/F042868); PK is a Senior Fellow of the British Heart Foundation; BR is supported by a Medical Research Council Career Development Award (G0700278).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC156

**Clinical evidence of spatial heterogeneity in heart rate adaptation and associated arrhythmic risk in the human ventricles**

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**Motivation:**

Adaptation of action potential duration (APD) refers to the slow change in APD following a sustained change in heart rate. The main function of this slow APD adaptation is to optimize the ratio of diastolic filling time to systolic ejection time (1). However, slow dynamics of adaptation of repolarization to changes in heart rate have been proposed as an arrhythmic risk biomarker (2). Heterogeneity in APD adaptation has been reported in animals (3,4), but has not been characterised in humans. In this study, we provide evidence of spatial differences in APD adaptation in the in-vivo left and right ventricles (LV/RV) in humans.

**Methods:**

Unipolar electrograms were simultaneously recorded at 20 endocardial sites covering apex to base on the RV septum and LV free wall of 7 patients with normal ventricles. Local time constants ($\tau$) for activation-recovery interval (ARI, as a surrogate of APD) adaptation were calculated following a heart rate change from sinus rhythm ($846.6 \pm 133.9$ ms) to $500$ ms cycle length, paced at RV apex. Computer simulations of the observed scenarios were carried out to investigate the effect of heterogeneity in APD adaptation in modulating dispersion of repolarization.

**Results:**

Adaptation dynamics in RV were spatially homogeneous in most patients (Fig A), but high inter-patient variability in time constants exists (mean $\tau$ range: 21.2 to 46.8 s). In LV, 3 patients showed longer time constants (slower adaptation) at base than apex (Fig B). These trends were also observed in the mean values of time constants (Fig C), with time constants between the base of LV and RV being significantly different (Fig D, Student’s T-test, p<0.05). Computer simulations show that marked gradients in adaptation dynamics enhance transient dispersion of repolarization in the human ventricles, providing a pro-arrhythmic substrate following changes in heart rate.

**Conclusions:**

We show in humans that: 1. Regional heterogeneity of repolarization occurs dynamically in the ventricles during adaptation to a change in heart rate; 2. Marked inter-patient variability is present in the maximum local repolarization gradients; 3. Computer simulations indicate that these spatial differences may be able to detect tissue’s propensity to develop transient conduction block after changes in heart rate, and that suitable substrates for reentry which may not be present at baseline may form dynamically due to this regional heterogeneity in APD adaptation.


Excitatory and inhibitory control of L-type Ca\(^{2+}\) current by pH in ventricular myocytes

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The modulatory effect of H\(^{+}\)-ions on the L-type Ca\(^{2+}\)-current (I\(_{\text{Ca,L}}\)) in mammalian ventricular myocytes is controversial, with reports suggesting either inhibition (Ref 1) or no effect (Ref 2). Significant modulation would imply that acid-base disturbances control excitation-contraction coupling in heart, at least partly by targeting I\(_{\text{Ca,L}}\). The sensitivity of I\(_{\text{Ca,L}}\) to experimental changes of pH\(_{i}\) and pH\(_{o}\) was investigated (whole-cell voltage-clamp), while measuring intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)), in rabbit & guinea-pig ventricular myocytes (similar results in both species). In rabbit myocytes exposed to 30 \(\mu\)M carbiprole (to inhibit Na\(^+\)/H\(^+\) exchange), reducing pH\(_{i}\) to 6.50 (Hepes-buffered superfusates) decreased I\(_{\text{Ca,L}}\), at a test voltage of -10mV, by -52.3 \(\pm\) 1.9%, and by -13.0 \(\pm\) 1.7 % at +20mV (n=7). In contrast, reducing pH\(_{o}\) from 7.20 to 6.80 (80mM Na\(^+\)-acetate superfusion) increased I\(_{\text{Ca,L}}\) by +73.8 \(\pm\) 9.1 % at a test-voltage of -10mV, but more modestly decreased it by -25.1 \(\pm\) 1.5 % at +20mV (n=14). The effect of pH\(_{i}\) on I\(_{\text{Ca,L}}\) was the same when perforated instead of whole-cell patch pipettes were used to control membrane potential (amphotericin B; n=8). When Ca\(^{2+}\)\(_{i}\) was buffered with 5M intracellular BAPTA, the excitatory effect of low pH\(_{i}\) was even more marked (+221.2 \(\pm\) 20.0 %) at -10mV, with no significant inhibition at +20mV (n=10). This result suggests that inhibition by low pH\(_{i}\) is secondary to a rise of Ca\(^{2+}\)\(_{i}\), while stimulation is a direct effect of intracellular H\(^{+}\) ions. We conclude that extracellular H\(^{+}\) ions inhibit while intracellular H\(^{+}\) ions can stimulate I\(_{\text{Ca,L}}\). In further experiments, low pH\(_{i}\) and pH\(_{o}\) effects were additive, tending to cancel when appropriately combined. They persisted after inhibition of CaM-kinase II (with 1.0 \(\mu\)M KN-93; n=8). These effects are consistent with fixed negative charge-screening by H\(^{+}\)-ions on both sides of the sarcolemma (altering the voltage-field local to Ca\(^{2+}\) channel proteins), with additional channel-block by H\(^{+}\), and Ca\(^{2+}\)\(_{i}\).

Action potential duration (APD) was also strongly H\(^{+}\)-sensitive, being shortened by low pH\(_{i}\) (-17.8 \(\pm\) 2.7 % at 90% repolarisation, n=9), but lengthened by low pH\(_{o}\) (+57.2 \(\pm\) 6.5 %, n=15). Results of reducing pH\(_{i}\) or pH\(_{o}\) while measuring I\(_{\text{Ca,L}}\) (nimodipine/Ca\(^{2+}\)-sensitive current) under action potential clamp control indicate that such effects on APD are caused mainly by H\(^{+}\)-induced changes in late Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\)-channel. Kinetic analyses of pH-sensitive channel-gating, when combined with whole-cell modelling, successfully predict the APD-changes, plus many of the accompanying changes in Ca\(^{2+}\)-signalling. We conclude that the pH\(_{i}\) versus pH\(_{o}\)-control of I\(_{\text{Ca,L}}\) will influence electrical and Ca\(^{2+}\)-dependent signalling during acid-base disturbances in the heart.

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This work is supported by the British Heart Foundation (RDV-J) and the National Institutes of Health (KWS)

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC157

A novel method to localize subcellular Ca\(^{2+}\) release using 3-D distance mapping

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In ventricular myocytes, L-type Ca\(^{2+}\) channels (LTCC) on the sarcoplemma (SL) and clusters of ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR) come in close proximity. Simulations of the SL permeate the cell, called T-tubules (TT). These allow functional coupling of the SL and SR, allowing a rapid global increase in cytosolic [Ca\(^{2+}\)]\(_{i}\) for the activation of contraction. However, not all RyR clusters are coupled, leading to temporal inhomogeneity of systolic Ca\(^{2+}\) release, which is more pronounced in larger mammals (Heinzel et al., 2002). Investigation of release, assigning events to coupled and uncoupled regions is currently limited by the temporal resolution of confocal microscopy to simultaneously monitor the 3D configuration of release sites and SL. Here we present a new method to investigate the subcellular activation of release of Ca\(^{2+}\) with proximity to TTs in pig ventricular myocytes. Whole-cell voltage clamp was used to elicit Ca\(^{2+}\) release by depolarizing steps at different frequencies (0.5 and 2 Hz) at 37°C. Cytochalasin D was used to inhibit contraction. Confocal line scanning of fluo-4 fluorescence was used to monitor Ca\(^{2+}\) transients during depolarizing steps from -70 mV to +10 mV at different frequencies. The temporal mid-point of the rising phase (TF50) was used to assess latency of release. SL membranes were fluorescently labeled with wheat germ agglutinin-Alexa594 and vertical image stacks were recorded for each cell. The 3-D TT geometry was assessed using the Euclidean distance mapping method. This method allowed the correlation of Ca\(^{2+}\) TF50 for each site in the line scan recording with distance to TT in 3D. A linear relationship was found for release between 0.5 and 3 \(\mu\)m from T-tubules at 2 frequencies: 0.5 Hz (R\(^2\) = 0.88; n\(_{\text{cells}}\)=7, N\(_{\text{pigs}}\)=5) and 2 Hz (R\(^2\) = 0.80; n\(_{\text{cells}}\)=7, N\(_{\text{pigs}}\)=5). This allowed the use of TF50 to map subcellular regions of Ca\(^{2+}\) release defined as coupled (<0.5 \(\mu\)m) and uncoupled (>1 \(\mu\)m). Regions of coupled release were found to reach T\(_{\text{F50}}\) within 19 ms and 14 ms, respectively for 0.5 Hz and 2 Hz stimulation. This resulted in 55% (0.5 Hz) and 49% (2 Hz) of coupled regions. Non-coupled release regions were found to reach T\(_{\text{F50}}\) within 22 ms and 17 ms, for 0.5 Hz and 2 Hz respectively. Hence 38% of regions were classified as non-coupled for both frequencies. This novel method shows 3-D distance mapping is a useful technique to localize subcellular Ca\(^{2+}\) release. This will be extended to allow investigation of spontaneous diastolic Ca\(^{2+}\) release events (sparks) from RyRs in different subcellular domains.

Instead of using a 3-D mapping method, this method allowed the correlation of Ca\(^{2+}\) TF50 for each site in the line scan recording with distance to TT in 3D. A linear relationship was found for release between 0.5 and 3 \(\mu\)m from T-tubules at 2 frequencies: 0.5 Hz (R\(^2\) = 0.88; n\(_{\text{cells}}\)=7, N\(_{\text{pigs}}\)=5) and 2 Hz (R\(^2\) = 0.80; n\(_{\text{cells}}\)=7, N\(_{\text{pigs}}\)=5). This allowed the use of TF50 to map subcellular regions of Ca\(^{2+}\) release defined as coupled (<0.5 \(\mu\)m) and uncoupled (>1 \(\mu\)m). Regions of coupled release were found to reach T\(_{\text{F50}}\) within 19 ms and 14 ms, respectively for 0.5 Hz and 2 Hz stimulation. This resulted in 55% (0.5 Hz) and 49% (2 Hz) of coupled regions. Non-coupled release regions were found to reach T\(_{\text{F50}}\) within 22 ms and 17 ms, for 0.5 Hz and 2 Hz respectively. Hence 38% of regions were classified as non-coupled for both frequencies. This novel method shows 3-D distance mapping is a useful technique to localize subcellular Ca\(^{2+}\) release. This will be extended to allow investigation of spontaneous diastolic Ca\(^{2+}\) release events (sparks) from RyRs in different subcellular domains.

PC158

Poster Communications

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

141P
Conduction velocity changes contribute to arrhythmogenicity in a murine model of catecholaminergic polymorphic ventricular tachycardia, RyR2-P2328S

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a potentially lethal familial disease characterized by bidirectional ventricular tachycardia (BVT), polymorphic VT (PVT), and ventricular fibrillation. Cardiac ryanodine receptor (RyR2) mutations associated with autosomal dominant forms of CPVT (Priori et al., 2001) result in increased diastolic sarcoplasmic reticular (SR) Ca²⁺ release (Jiang et al., 2002; George et al., 2003; Lehnart et al., 2004), but the mechanisms by which this might trigger and sustain fatal arrhythmias are incompletely understood.

ECGs were recorded from mice under terminal anaesthesia (ketamine 3.6 mg/10g + xylazine 0.16 mg/10g I.P.) Isoprenaline (iso, 2 mg/kg) and caffeine (120 mg/kg) challenge revealed a significant arrhythmic phenotype for homozygous (s/s) RyR2-P2328S mice (Goddard et al., 2008), with 21.2±9.7 (n=6) episodes of bigeminy, BVT or PVT per 10 min, compared to ±2 for WT or +/s mice (P<0.05), and 2.5±1.1 per 10 min (n=6) for s/s mice exposed to iso alone. No arrhythmic episodes were recorded under control conditions (n=12 in each case).

Similarly, PR intervals, QRS durations, QT intervals and heart rate (HR) were not significantly influenced by genotype under control conditions. Iso challenge increased HR by ~20% in each case (P<0.01), with no other significant electrocardiographic changes. However, iso and caffeine together markedly reduced HR and prolonged QT and corrected QT interval specifically in the s/s mice relative to control conditions and to WT (each P<0.01), suggesting a possible conduction velocity (CV) abnormality that was investigated further.

As shown in Fig. 1, CV was measured by fitting a regression plane to mean relative ventricular activation times recorded using a 64-electrode array in intrinsically beating Langendorff-perfused hearts, (n=6 in all cases). Multiple-r values of such planes ranged from 0.85 to 0.98, consistent with an acceptable planar approximation.

Fig. 2 shows that CVs were indistinguishable in WT, +/s and s/s ventricles before pharmacological manipulation and were increased by iso in WT and s/s. However, the iso + caffeine combination markedly reduced CV in the s/s relative to control conditions, iso exposure and WT. These findings were corroborated by conventional 20 MΩ intracellular glass microelectrode AP recordings. Maximum AP upstroke rate was reduced specifically in s/s hearts following application of iso + caffeine.

We thus correlate the increased arrhythmic tendency of RyR2S/S hearts following adrenergic and caffeine challenge with significantly decreased epicardial CV and AP upstroke velocity.
PC160

Electrical remodeling of potassium currents in right atrial myocytes from sheep persistent atrial fibrillation model

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Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia observed in clinical practice. Persistent atrial fibrillation has been associated with structural and electrical remodeling. The objective of this study was to characterize the remodeling of potassium currents (especially the inward rectifier IK1, the transient outward Ito and the ultra-rapid delayed rectifier I Kur) in a sheep model of persistent AF. We studied whole cell currents with the patch-clamp technique in right atrial myocytes isolated from 5 sheep with persistent AF, compared to 11 animals in sinus rhythm. AF animals were instrumented with an atrial and ventricular pacemaker using a transvenous approach. Pacemaker insertion at the shoulder was carried out under general anesthesia with isoflurane (1%) after pre-medication with ketamine (10 mg.kg-1) and xylazine (0.2 mg.kg-1) intramuscular.

First of all, the density of the inward rectifier IK1 (considered as the Ba2+ 500μM sensitive current) was greater in AF animals (-3.37±0.34 pA/pF vs -6.1±0.59 pA/pF at -120mV, p<0.0001). The reversal potential and the potential at maximal density were not different between control and AF animals. We considered the ultra-rapid delayed rectifier current as the 4-AP 50μM sensitive current and the transient outward current as the 4-AP 5μM sensitive current. The density of the 4-AP 50μM sensitive current tended to be smaller in the AF group (0.92±0.14 pA/pF vs 0.52±0.12 pA/pF at +60mV, p=0.186), without any difference in the time constant of inactivation (241±43 msec vs 438±99 msec in SR and AF animals, respectively, NS). The density of the 4-AP 5μM sensitive current was significantly smaller in AF atrial cells (1.4±0.19 pA/pF vs 0.86±0.18 pA/pF at +60mV, p<0.05), without any difference in the time constant of inactivation (85±23 msec vs 68±12 msec in SR and AF atrial cells, respectively, NS). Persistent AF induces electrical remodeling of potassium currents in sheep. The ultra-rapid delayed rectifier and the transient outward currents are decreased, which can induce the occurrence of a triangular action potential (AP) which poorly adapts to heart rate changes. The inward rectifier current is increased, which shortens the AP, can induce a greater excitability of atrial cells and promote re-entry.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC161

Effects of detubulation on excitation-contraction coupling in sheep atrial myocytes

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Transverse (t-) tubules are essential for facilitating the synchronous increase in intracellular calcium giving rise to the calcium transient in mammalian ventricular myocytes. It has recently been established that atrial cells of the sheep possess a functional t-tubule network (Dibb et al., 2009) unlike atrial cells of rodents (reviewed by Bootman et al., 2006). Importantly, atrial t-tubules are lost in sheep models of cardiac disease (Dibb et al., 2009, Lenaerts et al., 2009). Here we examine the functional importance of t-tubules for excitation-contraction coupling in sheep atrial cells. Healthy adult sheep were killed in accordance with The Home Office Animal (Scientific Procedures) Act, 1986. Myocytes were enzymatically isolated from the left atrial appendage. Formamide was used to detubulate the myocytes (Brette et al., 2002). T-tubules were visualised using di-4-ANEPPS. Cells were voltage-clamped using the perforated patch technique and stimulated at 0.5Hz. Intracellular calcium was measured using Fura-2 AM. Data are expressed as mean ± SEM and statistical significance was considered to be when p<0.05.

Confocal imaging using di-4-ANEPPS confirmed that treatment with 4% formamide could successfully uncouple t-tubules from the sarcolemma in sheep atrial myocytes. Detubulation resulted in a decrease of cell capacitance (104± ± 6 4 μF, p<0.01, n=12-14) without a change in cell length (135 ± 7 vs. 137 ± 4μm, p>0.05, n=6-8) or width (20 ± 3 vs. 23 ± 4μm, p>0.05, n=6-8). Peak I c4Ca was reduced in detubulated myocytes (1.5±0.2 vs. 0.4±0.1 pA/pF, p<0.001, n=11-13) and accompanied by a 53% decrease in calcium transient amplitude. In addition, the calcium transient had a slower time to peak (106±15 vs. 275±9 ms, p<0.01, n=10-13), suggesting asynchronous calcium release.

These data show that sheep atrial t-tubules are functionally important for E-C coupling. T-tubules constitute 40% of cell capacitance with ~70% of the total Ic4Ca residing in these structures. Importantly, formamide treatment qualitatively mimics alterations in atrial calcium handling previously observed in a sheep model of heart failure where t-tubules are lost. This suggests t-tubule loss has important functional consequences in cardiac disease.


This work was supported by the British Heart Foundation.

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PC162

Two pathways to sinoatrial node dysfunction in heart failure

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The sinoatrial node (SAN) is the primary pacemaker of the heart. SAN dysfunction is associated with heart failure (HF), and bradyarrhythmias are responsible for a substantial proportion of deaths of HF patients. Ion channels and their associated subunits are key to the pacemaker function of the SAN, and we investigated whether there are changes in ion channel gene expression in the SAN in HF. Three animal models were
studied: (i) a rabbit model of volume and pressure overload (caused by destruction of the aortic valve and banding of the aorta under ketamine HCL (50 mg/kg body weight, i.m.) anesthesia, followed by temgesic (15 mg/kg s.c.); (ii) a rat model of pulmonary hypertension (PHT; caused by subcutaneous injection of monocrotaline (produced by a single injection of 60 mg/kg monocrotaline (MCT) solution s.c. into the interscapular region and killed under sodium pentobarbiturate (45mg/kg) anesthesia)); and (iii) a rat model of myocardial infarction (MI; caused by ligation of the proximal left coronary artery under ketamine HCL and Xylazine (100mg/5mg/kg body weight, i.p.) anesthesia). All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, revised 1985). In the rabbit and rat MI models, there was a decrease in the intrinsic heart rate (evidence of SAN dysfunction; measured in Langendorff heart experiments). Tissue was sampled from the right atrium and SAN, and expression of mRNAs for ion channels and related proteins was measured using quantitative PCR. Expression of between 33 and 80 mRNAs was measured.

During HF, a relatively small number of significant changes in expression were observed in atrial muscle (6, 27 and 7% in rabbit, rat PHT and rat MI models), but a larger number was observed in the SAN (48, 58 and 40% in rabbit, rat PHT and rat MI models). In the rabbit and rat PHT models, the HF-related bradycardia can perhaps be explained by a downregulation of expression of HCN4 (of -87 and -41%), Cav1.2 (of 51 and -70%) and NCX1 (of -46 and -38%), all of which carry inward current; measured in Langendorff heart experiments). Tissue was sampled from the right atrium and SAN, and expression of mRNAs for ion channels and related proteins was measured using quantitative PCR. Expression of between 33 and 80 mRNAs was measured.

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**PC163**

**How does ageing modulate intracellular Ca homeostasis in the sheep atria?**

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Ageing is an important risk factor for the development of the most common cardiac arrhythmia, atrial fibrillation (AF). The prevalence of AF is set to increase as society ages. Despite the fact that AF may arise due to perturbations in Ca handling, little is known regarding how intracellular Ca is affected by ageing. We have previously shown altered Ca handling in the aged atria. Here we have investigated how changes in expression levels and functional properties of key proteins could account for these changes. Young adult (18 months) and old sheep (>8 years) were euthanized (200 mg/kg intravenous pentobarbitone) and myocytes isolated from the left atrium by enzymatic digestion. Atrial myocytes were stimulated at 0.5Hz under voltage clamp control using the perforated patch clamp technique at 37°C. Changes in intracellular Ca were measured using Fluo-5F AM. Data are presented as mean ± SEM for n cells and statistical analysis was performed using a t-test or a Mann Whitney Rank Sum Test.

Ageing decreased the peak of the L-type Ca current (\(I_{Ca-L}\)) by 19% (2.26 ± 0.14 vs. 1.84 ± 0.11 pA/pF, \(p<0.05, n=47-67\)) which is likely to play a role in the 24% decrease in Ca transient amplitude. An age associated reduction in SERCA function was accompanied by a reduction in SERCA protein levels with no change in phospholamban as shown by western blotting (\(p<0.001\). Surprisingly sarcoplasmic reticulum (SR) Ca content was increased (83.4 ± 3.48 vs. 101.9 ± 4.40 μmol/l, \(n=35-50\) cells, \(p<0.01\)). We sought to investigate SR function by plotting the relationship between Ca transient amplitude and SR Ca content (Trafford et al., 2001;Kettlewell et al., 2005). This was achieved by depleting the SR with caffeine and refilling to different levels by altering the number of recovery pulses before assessing SR Ca content again by caffeine application. This protocol was repeated until Ca transient amplitude had returned to steady state. A 3 parameter power function was fitted to the relationship between Ca transient amplitude and SR Ca content. Ageing resulted in a rightward shift of this relationship (\(p<0.001\)) suggesting a decrease in the sensitivity of Ca release. To investigate potential roles for decreased \(I_{Ca-L}\) vs. decreased ryanodine receptor (RyR) Ca release capacity we divided Ca transient amplitude by peak \(I_{Ca-L}\) and expressed this as a function of SR content. Ageing still resulted in a rightward shift of this relationship suggesting roles for both \(I_{Ca-L}\) and RyR. In summary, decreased \(I_{Ca-L}\) may underlie the age associated reduction in Ca transient amplitude however it remains to be determined if reduced \(I_{Ca-L}\) can fully account for reduced Ca transient amplitude in the presence of increased SR Ca content. A decreased Ca release capacity of RyR may offset decreased SERCA function in determining SR Ca content in the aged atria.


This work was supported by the British Heart Foundation

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**PC164**

**Gene expression in glycol tRNA synthetase (GARS) related neurodegeneration**

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Background:

Gene expression in glycol tRNA synthetase (GARS) related neurodegeneration
GARS gene encodes Glycyl-tRNA Synthetase, which is an enzyme linking tRNA to glycine. Mutation in GARS have been shown to cause distal spinal muscular atrophy (dSMA) and Charcot-Marie-Tooth disease type 2D (CMT2D). In both conditions, the prominent early feature is wasting and weakness of the muscle of the hands and feet caused by degeneration of peripheral nerves (1). However, the function and relative role of Gars in triggering neurodegeneration has not been discovered. The aim of this study is to identify the correlated genes differently expressed between wild type and mutant of the Gars using an in vitro stable neuronal cell line model and in vivo mouse tissues.

Material and method:
Mouse cDNA: wildtype Gars and mutant(2) cDNA were tagged with an OneStrep tag and were cloned into pTightPuro vector. We then used Lenti-XTM Tet-On® advanced inducible expression system to generate mouse stable cell lines, which involves packaging these cDNA plasmids into lentiviral particles and cotransducing with the regulator TetOn virus into target NSC-34 cells. Monoclonal cell lines were screened with immunofluorescence and western blotting to confirm the expression of the transduced gene. RNAs were extracted from the expressed cell lines and were then carried out Affymetrix microarray study. Positive targets were validated using quantitative real-time PCR on both cell lines and different mouse tissues.

Result:
NSC-34 cells were successfully transduced as indicated by the expression of the Strept tag, which was tested using Immunofluorescence and western blot. When comparing wildtype and mutant type, microarray data showed a list of genes expressed differently with significant fold changes. Subsequent validation using Q-PCR showed a general correlation to microarray data with higher fold changes in some individual targets.


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The project is funded by Medical Research Council.

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PC165

Development of physiologically correct neural inputs for an in vitro model of skeletal muscle
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The ability to engineer physiologically correct in vitro models of skeletal muscle is likely to play an important role in the future investigation of skeletal muscle development, physiology and pathology. Although current models develop a physiological architecture and contractile properties similar to those of the nascent tissue, there is now a drive to promote maturation towards a mature phenotype, which necessitates integration of a neural input into the model.

This work seeks to establish a neural input for an established skeletal muscle model through the integration of primary motoneurons with cultured myotubes. As well as promoting maturation towards an adult phenotype, reliable neuromuscular junction (NMJ) formation in 3D culture could have substantial benefits in the study of neuromuscular disease and the testing of novel therapeutic agents.

Methods for establishing the muscle model are detailed elsewhere (Cheema et al., 2003) and rely on the inherent ability for cells seeded in a 3D matrix to self orientate. Within this model, contraction of the seeded cells against uniaxial fixed points leads to formation of isometric tension sufficient to promote the reorganisation of the cells along lines of strain. Constructs were cultured with primary embryonic rat motoneurons for 14 days before being prepared for analysis.

Images from stained sections of the constructs demonstrate the improved functional architecture of the 3D muscle constructs compared to 2D and the similarity to that observed in vivo muscle controls (n=9). Close association of developing neurites with underlying myotubes is clear and there is evidence of formation of structures in vitro that closely resemble NMJs in vivo. Gene expression analysis suggests a 2-3 fold increase in expression of markers of muscle maturation, such as myosin heavy chain isoforms, troponin and acetylcholine receptor subunits, in 3D co-cultures compared with muscle only controls (n=3).

Current efforts are focused on improving the incidence of synapse formation and further improving myotube-motoneuron interaction in vitro. Once optimised, this development in muscle modelling should have significant repercussions in the future investigation of neuromuscular interaction.


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PC166

Melatonin Inhibits Membrane Depolarisation-Induced Calcium Signals in Cultured Rat Trigeminal Ganglion Neurons
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Acute or chronic orofacial pain is a symptom associated with disorders of oral soft and hard tissues, and represents a common problem of modern society. Trigeminal ganglion neurons, orofacial nociceptors, are primary sensory neurons that have proven to be an efficient cellular model for studying orofacial pain mechanisms. Free intracellular calcium is a ubiquitous second messenger being involved in a wide range of neuronal functions including control of excitability and neurotransmitter release, and nociceptive signal transmission. We have investigated, by considering intracellular calcium concentrations as a key signal for nociceptive transmission, the effects of melatonin on membrane depolarization-induced calcium signalling in rat trigeminal ganglia neurons. Trigeminal neurons were cultured on glass coverslips following enzymatic digestion and mechanical agitation, and loaded with the cal-

145P
Dynamics of CA1 and subicular neurone firing during theta and slow-wave rhythm transitions in the anaesthetized rat

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Neurones within the hippocampus code for aspects of episodic memory such as encountered objects, the position and motion path of the animal, past and future behaviour (1). The processing of this hippocampal information is widely accepted to be orchestrated in a phase-dependent manner during oscillatory states such as theta and slow-wave rhythms (2,3). The major target for hippocampal CA1 output is the subiculum, however, despite its strategic position we know comparatively little regarding how subicular neuronal activity is organized with that of CA1. To address this we recorded simultaneous activity from CA1 and subiculum during theta and slow-wave in the urethane-anaesthetized Wistar rat (1.5g/kg; 30% w/v i.p.; n=6). Separate recording electrode arrays (each with thirty-two 413 μm² contacts over 4 shanks, spaced 50 μm vertically and 200 μm horizontally; Axiolab-5x0-200-413, NeuroNexusTech, USA) were placed in pyramidal layers of dorsal CA1 and subiculum. Spontaneous spikes and local field potentials were recorded from each electrode for at least one hour (Recorder64, Plexon, USA). Recordings showed predominant theta activity from CA1 and subiculum during theta and slow-wave, with the occasional transition to slow-wave (c.25% of period), that is, not at theta 'trough'; and (b) that the potential ‘read-out’ of information from subiculum during slow-wave may be facilitated by a switch in spike firing of individual subicular neurones from tonic to bursting patterns.

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Poster Communications

Distributed control strategies for conductance regulation in dendrites

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A microscope image of a typical neurone points to an interesting logistical problem for mechanisms that regulate intrinsic membrane properties. Dendritic arbour is complex, with long, thin processes and varicosities that effectively compartmentalise biochemical and electrical signals. Distributions of membrane conductances therefore exert local effects as well as influencing the electrical properties in entire cells. Moreover, feedback signals representing the state of a particular location in a cell will only reflect local conditions. It is hard to imagine how regulatory control can be orchestrated centrally (at the soma, for example) such that the target intrinsic properties are satisfied in all compartments of the cell simultaneously, yet most existing models of activity-dependent regulation of intrinsic properties assume that changes are cell-wide and uniform within a cell [1]. This problem, which has received a great deal of attention in the context of synaptic homeostasis [2], has not been not been extensively investigated in the context of homeostatic control of intrinsic properties [3]. In this work we examine the effect local homeostatic mechanisms have on global intrinsic properties and on conductance distributions in dendrites using a multi-compartment conductance-based model. We find that simple rules can generate heterogeneous conductance distributions in morphologically reconstructed cells, and investigate the effect of noise on the performance of several biologically plausible homeostatic control mechanisms. Noise is found to distinguish the utility of different feedback control mechanisms (such as linear integral controllers and non-linear ‘bang-bang’ controllers) under simple assumptions, and this has important implications for the nature of biological homeostatic mechanisms that give rise to experimental data.
Establishment of a motoneuron-myotube co-culture in a 3D fibrin gel model of skeletal muscle

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Introduction. There is currently no truly biomimetic in vitro model of skeletal muscle incorporating a functional neuronal input analogous to that seen at the in vivo neuromuscular junction. Establishment of such a model would allow detailed study of neuromuscular biology and skeletal muscle in health and disease whilst greatly reducing reliance on the use of in vivo models. Fibrin cast skeletal muscle shows similar physiological and contractile characteristics to in vivo muscle including the ability to generate force in response to electrical stimulation (1). We have now further developed the fibrin muscle model by the co-culture of primary rat muscle-derived cells (MDCs) and primary rat motoneurons (MNs), with the aim of generating an engineered muscle construct with functional neuronal input.

Methods. Fibrin cast skeletal muscles were engineered as described previously (1). Rat MDCs were isolated from the hind limbs of P1 Sprague-Dawley rat pups by collagenase digestion and cultured on fibrin gels. When the MDCs became confluent MNs isolated from the ventral horn of spinal cords from Sprague-Dawley rat E14 embryos were plated at 50,000 cells per gel. Gels were fixed 7 days post MN-seeding and fluorescence immunocytochemistry was used to label the myotubes (desmin), the motoneurons (MAP2, SV2, 2H3), and the Acetylcholine receptors (AChR: TexasRed-Bungarotoxin (BTX)).

Results. Microscopic observations reveal formation of a tight bundle of aligned multinucleate myotubes analogous to that seen in vivo skeletal muscle. Functional maturation of the myotubes was evident, as myotubes developed the striated appearance characteristic of skeletal muscle and spontaneous contractions were observed (n=4). MNs survive within the muscle fibrin cultures for at least 7 days (n=2), and MN survival was shown to be greater in gels seeded with 200,000 MDCs compared to 400,000 MDCs (11.5 vs. 3.65 MN cells/field of view, n=2). BTX clustering and colocalisation with MN markers (n=3) was evident.

Discussion. Using an established model of skeletal muscle, we have added extra complexity through the addition of a neuronal input. Fibrin cast skeletal muscles can be rapidly engineered together with MN, producing contractile neuromuscular constructs. No only do MNs survive in the co-culture system, they also induce AChR clustering at sites of contact indicating physiological interactions between the two cell types. These data are the first step towards the formation of organised NMJs. Establishment of a 3D muscle-MN culture system will be of great benefit to the study of NMJ formation, maturation, and function and will reduce reliance on animal models for such studies.


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PC169

The Endogenous Salivary Peptide Opiorphin Inhibits Ca2+ Signalling in Rat Primary Sensory Neurones

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Evidence from recent in vivo behavioural pain studies suggests that the inhibition of enzymes responsible for the inactivation of enkephalins, endogenous opiate receptor agonists with potent analgesic properties, are potential targets for effective endogenous modification to obtain strong, safe and long lasting analgesia. However, the cellular mechanisms by which the inhibitor of multiple enkephalin degrading enzymes are acting, and their possible peripheral antinociceptive effects is not studied yet. The aim of the present study was to investigate the effects of opiorphin, a natural peptide inhibitor of enkephalin degrading-enzyme, on peripheral nociception by investigating its action on intracellular Ca2+ signalling in rat dorsal root ganglion (DRG) neurones. All experimental procedures were approved by the Institutional Ethics Committee. Following enzymatic digestion and mechanical agitation the DRG neurones were cultured on coated coverslips and loaded with 1 umol Fura-2 AM. Intracellular calcium responses were quantified by the changes in 340/380 ratio for individual DRG neurons using fluorescence imaging system consisting of CCD camera coupled to an inverted microscope with a 40x (1.30 NA) objective. Brief exposure to high KCl (30 mM) was used to stimulate intracellular calcium signals, and effects of opiorphin was investigated. All data were analyzed by using unpaired t test, P <05 defining statistical significance. Acute application of 10, 30 or 100 uM opiorphin failed to produce any significant change in KCl (30 mM)-induced elevation in cytosolic calcium levels (each tested on cells from three different culture dishes). Treatment with opiorphin for 30 minutes caused a complete inhibition of cytosolic calcium responses to KCl (30 mM). The mean 340/380 ratio was increased from baseline level of 0.79±0.05 (n=67) to 1.2±0.06 with KCl exposure (30 mM KCl, P<0.05, n=67) which remained without significant change at 5th minute but abolished after 30 minute treatment with 100 uM opiorphin (30 mM KCl + 100 uM opiorphin: 0.78±0.07, n=55). We report for the first time in these peripheral nociceptive sensory neurones that opiorphin inhibits membrane depolarisation-induced calcium signalling. Although this effect takes time, considering the fact that opiorphin advantages with respect to the abuse liability and drug tolerance, this agent or its proper derivatives are promising candidates for treatment of pain conditions.

PC170

Evidence from recent in vivo behavioural pain studies suggests that the inhibition of enzymes responsible for the inactivation of enkephalins, endogenous opiate receptor agonists with potent analgesic properties, are potential targets for effective endogenous modification to obtain strong, safe and long lasting analgesia.
Intravital two-photon imaging of mice infected with *Trypanosoma brucei* shows parasites moving in the subarachnoid space and brain perivascular spaces


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*Trypanosoma brucei*, the protozoan that causes African sleeping sickness, can be eliminated from the blood by treatment with available drugs, but in the later stages, after trypanosomes have invaded the brain, current treatments are less successful. To better understand the process of invasion, we have used two-photon microscopy to image trypanosomes in vivo in the meninges and superficial brain through the thinned skull of mice. CD1 mice were infected i.p. with the GVR35 strain of *T. brucei*, and 3 to 28 days later were injected intravenously with fluorescent markers: dextran–rhodamine to label blood, and the diamidine derivative DB75 which is transported into trypanosomes [1]. The mice were then anaesthetised with Hypnorm/Hypnovel (0.05/0.05 mL per gm body weight, i.p.), the anaesthesia being maintained by supplying oxygen carrying gradually increasing amounts of isofluorane (0-2%). A plate with a hole 5 mm in diameter was glued to the skull, and, held by the plate, the skull was thinned over an area about 3 mm in diameter to a thickness of 20-30 μm [2]. Extravascular trypanosomes were observed in clusters in the subarachnoid space centered at a depth below the skull of 22 ± 7 μm (mean ± SD, 6 mice, Figure 1A,B) and they moved mainly within restricted spaces about 30 μm in diameter to a thickness of 20-30 μm (mean ± SD, 6 mice, Figure 1C). Occasional putative trypanosomes were observed at depths up to 189 μm below the skull; these were closely associated with vessels penetrating into the cortex. Trypanosomes were observed in the subarachnoid space between 7 and 28 days after infection. Over this period the number of trypanosomes per unit area of subarachnoid space did not vary significantly (mean 165 ± 148 μm² in 11 mice) although neurological symptoms develop later and progressively. It is not clear how the trypanosomes reached the subarachnoid space. Early in brain invasion, trypanosomes are found in the choroid plexus, circumventricular organs and CSF, and there is bulk fluid flow from there to the subarachnoid space [3, 4]. But it has also been found that trypanosomes interact with and cross monolayers of endothelial cells cultured from brain microvessels [5]. Although, in vivo, we often saw intravascular leukocytes adhering to the endothelium of pial venules, we did not observe trypanosomes doing this. However, at one site, in one mouse, we twice observed a brief (<5 sec) release of blood that included a trypanosome.

This work was financially supported by The Scientific and Technological Research Council of Turkey (TUBITAK Project No: 1105140).

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tion induced [Ca2+]i in the SHR (F/F0: 1.54 ± 0.11, n=12) when compared with the empty vector transduced neurons (F/F0: 2.03 ± 0.08, n=5, p<0.05). NNOS specific inhibition by N-[(4S)-4-Amino-5-[(2-aminoethyl)[amino] pentyl]-N'-nitroguanidine (AAAN) reversed this response in transfected SHR with Ad.PRS-NNOS/mCherry (F/F0 from 1.54 ± 0.11 to 2.14 ± 0.17, n=12, p<0.01). These results show that cardiac sympathetic Ca2+ handling is impaired in young SHR before the actual onset of hypertension. Moreover, artificial up-regulation of cardiac sympathetic nNOS via gene transfer can directly attenuate intracellular Ca2+, and may provide a novel method for modulation of cardiac sympathetic neurotransmission.


This work was supported by British Heart Foundation.

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PC173

Cellular mechanism(s) of action of a water soluble extract of M. Charantia in inducing cell death on different cancer cell lines

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A multitude of plants have been used extensively for the treatment of cancers throughout the world. Much research has been focused on the scientific evaluation of several phytochemicals from the traditional plant Momordica charantia (M charantia) which has been used frequently as an anti-cancer agent. In a previous communication (Gunasekar et al, 2010), we have shown that the crude water soluble extract of M. charantia can evoke both time and dose dependent effects on cancer cell death. This study now investigated the cellular mechanism(s) whereby the crude water soluble extract of M. charantia can elicit cell death employing 1321N1, Gos-3, U87-MG, Weri Rb1, Sk Mel, Corl -23 compared to normal healthy L6 muscle cell line. This study measured the release of cytochrome-c, the activities of caspase-3 and caspase-9 and intracellular free calcium concentrations [Ca2+]i in the different cell lines following stimulation with 800 μg/ml of the crude water soluble extract of M. charantia for 24 hrs.

Initial results have shown that the crude water soluble extract of M. Charantia can evoke significant (Student's t-test; p<0.05) increases in the activities of both caspase-3 and caspase-9 compared to untreated cell lines over the same period. Typically, mean ±SD values for caspase-3 activity (pmol AMC min1mg protein1) were 0.42 ± 0.21, 0.37 ± 0.17, 0.41 ± 0.26, 0.51 ± 0.32, 0.64 ± 0.37, 0.67 ± 0.43, 0.34 ± 0.21, n = 12 in untreated cell lines compared to 0.84 ± 0.42, 0.79 ± 0.36, 0.77 ± 0.41, 0.86 ± 0.39, 0.95 ± 0.49, 0.83 ± 0.47, 0.62 ± 0.36, n = 12 in the following treated cell lines 1321N1, Gos-3, U87-MG, Weri Rb1, Sk Mel and Corl-23, respectively. The activity was much smaller in L6 cell lines. Similarly, the crude water soluble extract of M. Charantia can elicit significant (p<0.05) increases in cytochrome-c release in the six different cancer cell lines compared to untreated cells. The results also show that the crude water soluble extract of M. Charantia (800 μg) can evoke significant (p<0.05) and time-dependent increases in [Ca2+]i in all the six cancer cell lines employed in this study over a duration of 420 min compared to basal [Ca2+]i at the start of the experiment (0 min) and [Ca2+]i in each respective untreated cell line incubated alone in the medium for 420 min. The results also show that the extract had little or no significant effect on L6 skeletal muscle cell line. Mean ±SD basal [Ca2+]i in this series of experiment was 0.17 ± 0.09 ratio units (intensity), n = 82. The results indicate that the crude extract of M. charantia can exert its anti-cancer effect (cell death) on cancer cells via damage of cell mitochondria body resulting in elevation in such cellular mediators as [Ca2+]i, caspase-3 and caspase-9 and release of cytochrome-c.


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PC174

Leptin activates intracellular calcium signals in isolated rat dorsal root ganglion neurons

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Central and peripheral administration of leptin has been suggested to cause proalgesic actions but the mechanism of action is not clear. This study investigate the effects of leptin on intracellular calcium, ([Ca2+]i), levels in isolated rat dorsal root ganglion (DRG) neurons, with the aim of exploring possible involvement of leptin-mediated signalling in peripheral nociception.

DRG neurons were isolated from neonatal rats, plated on poly-d-lysine-coated coverslips. Isolated DRG neurons were loaded with 1 μmol Fura-2 AM and Ca2+ responses were assessed by using the fluorescent ratiometry. Fura-2 loaded DRG cells were excited at 340 and 380 nm, and emission was recorded at 510 nm by using digital microscopic calcium imaging system. Changes in free intracellular calcium concentrations were determined by the change in 340/380nm ratio in individual DRG neurons. All data were analyzed by using unpaired t test, with a one-tailed P level of <0.05 defining statistical significance.

Leptin caused increase in [Ca2+]i in a dose dependent manner. The mean 340/380 nm ratios were (baseline 100.0±0.0% vs leptin): 104.6±2.4% (n=18 cells) and 129.4±3.9% (10 μM Leptin, n=27 cells), following addition of respective concentrations of leptin. The PKC inhibitor chelerythrine chloride significantly decreased the leptin (1μM)-induced calcium responses (from 100.0±0.0% to 56.7±5.4%, n=27 cells). After depletion of thapsigargin-sensitive calcium stores by treatment of cells with thapsigargin (TG) attenuated but not completely blocked the subsequent calcium response to 1μM Leptin (TG +1μM Leptin = 106.1±2.4% (n=18 cells) vs. 1μM Leptin = 119.5±3.9% (n=27 cells). In sum we found that leptin mediate peripheral nociceptive signalling through mechanism that involves PKC-dependent changes in intracellular calcium levels in rat pri-
mary sensory neurons. Results from this study indicates that in addition to mediating the sensing of energy reserves in the body, leptin involved in the sensing process of pain.

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**PC175**

**Kainate-evoked glutamate secretion is reduced by ethanol in rat hippocampal astrocytes**

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The central nervous system is a major target for alcohol and its consumption has long been associated with brain damage. Ethanol may have several cellular targets in different brain areas, where ethanol may influence glutamatergic excitatory transmission. In this study we have used rat hippocampal astrocytes in culture to investigate the effect of ethanol on kainate-induced glutamate secretion and on intracellular free Ca²⁺ concentration ([Ca²⁺]c). Cell cultures were prepared by digestion of hippocampi with papain, followed by mechanical disaggregation, employing previously described methods (González et al., 2006). Astrocytes were cultured in medium consisting of DMEM supplemented with foetal bovine serum (10%), penicillin (20000 IU), streptomycin (20 mg/mL) and MITO+ (0.1%), at 37°C in a humidified incubator (5% CO₂). Glutamate release was monitored using an enzyme-linked system by which, in the presence of glutamate, glutamate dehydrogenase reduces NADP⁺ to NADPH. NADPH fluoresces when excited with UV light (Rodriguez-Moreno and Sihra, 2004). [Ca²⁺]c was monitored by single cell imaging analysis of fura-2-loaded cells. Our results show that kainate (10 μM to 500 μM), also blocked ethanol-inhibition of glutamate release from astrocytes. Preincubation of astrocytes in the presence of ethanol (1 mM-50 mM) induced a concentration-dependent inhibition of glutamate release caused by stimulation of cells with 100 μM kainate. Inhibition of alcohol-dehydrogenase, by preincubation of astrocytes in the presence of 4-methylpyrazole (1 mM), abolished ethanol-induced inhibition of glutamate release in response to kainate. On the other hand, preincubation of astrocytes in the presence of the antioxidant cinnamatinin B-1 (10 μM), also blocked ethanol inhibitory action on glutamate release in response to kainate. Stimulation of astrocytes with kainate (100 μM) induced an initial increase in [Ca²⁺]c followed by a progressive decrease. Ethanol (50 mM) reduced Ca²⁺ mobilization in response to kainate, whereas cinnamatinin B-1 reversed the inhibitory action of ethanol on Ca²⁺ mobilization by kainate. Our results are consistent with an inhibitory action of ethanol on glutamate secretion from hippocampal astrocytes. The inhibitory action of ethanol is probably due to its oxidative metabolism, and may involve reactive oxygen species production and a lower Ca²⁺ mobilization by kainate. Taking into account the pivotal role that astrocytes play within the central nervous system, especially in relation to neurons, the negative effects of ethanol on the release of glutamate might affect neuron-glia communication in the hippocampus, which might lead to functional defects in the brain.


Funding for this study was provided by Plan Regional de Investigación Sanitaria 2010 (PRIS10014) and Junta de Extremadura-FEDER (GR10010).

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**PC176**

**Activation of enteric glia during the colonic migrating motor complex (CMMC) in mice**

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Introduction: Enteric glial cells (EGCs) are astrocyte-like cells found in the enteric nerve plexuses. EGCs can modulate neural activity and electrophysiological events that have been shown to underlie propulsion of fecal pellets in the mouse (Heredia et al., 2010). The aim of this study was to determine whether EGCs are activated during the CMMC. Methods: Adult C57/BL6 mice were killed by inhalation of 5% isoflurane in 97% O₂ + 3% CO₂ followed by cervical dislocation. The entire colon was removed and pinned serosal side up and perfused with oxygenated Krebs’ solution at 37°C. Strips of longitudinal muscle were removed to expose myenteric ganglia and the tissue loaded with Fluo-4. Ca²⁺ imaging was used to examine the activity of myenteric neurons and EGC during the CMMC (Bayguinov et al., 2010). CMMCs were evoked either by stimulating the mucosa under the recording site with puffs of nitrogen or by brushing the mucosa at the anal end of the colon (Bayguinov et al., 2010). Following Ca²⁺ imaging experiments, tissues were dual-stained for neuronal Nitric Oxide Synthase (nNOS) and S-100. Results: S100 staining revealed a dense glial cell network around myenteric neurons; whereas, NOS immunoreactivity stained ~40% of all neurons in the myenteric ganglia. Glia loaded with Fluo-4 looked like tear dropped or triangular shaped cells that formed caps on the apical parts of neurons; whereas, their processes formed distinctive halos around myenteric neurons. Between CMMCs, Ca²⁺ activity in EGCs was variable, with most (83%, n=4) cells remaining quiescent, although slow fluctuations in Ca²⁺ were observed in a small number of cells. In contrast, many varicose nerve fibers and myenteric neurons, including NOS-positive inhibitory motor neurons, displayed uncoordinated fast Ca²⁺ transients (frequency 1.3 ± 0.2Hz). During CMMCs, which had duration of 27.9 ± 3.0 s, many varicosities and neurons displayed coordinated, prolonged phasic bursts of activity, while NOS-positive inhibitory motor neurons decreased their activity (Bayguinov et al., 2010). However, during a CMMC, EGCs exhibited a coordinated sustained, slow rise in calcium (duration: 24.2 ± 1.5 s; n=4) that slowly declined following the CMMC. Calcium transients in EGCs, which were observed to propagate along their processes, correlated with net increases in neuronal activity, as well as varicosities that were closely apposed to or upon the
glial cells. In the presence of TTX (1μM), high KCl (50mM), spritzing substance P (1μM) or sodium nitroprusside (10μM; nitric oxide donor) activated the glial cell network. Conclusion: EGCs are activated presumably by excitatory neurotransmitters during the CMMC, and possibly by nitric oxide between complexes. However, their role in modulating neuronal activity needs to be further elucidated.


Funding: National Institute of Health (USA) NIDDK R01 DK45713.

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PC177

Some observations on the innervation of the female rat anal sphincters

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The aim of this study was to examine the innervation of the rat sphincters of faecal continence. Female Wistar rats were anesthetised with urethane (1.5 g. kg-1 i.p.) and the femoral vein cannulated for administration of supplemental anaesthetic. The anal canal was arranged as an in vivo ring preparation of both external anal sphincter (EAS) and internal anal sphincter (IAS) both of which were connected to a single force transducer. All data are normally distributed, expressed as mean ± S.D. and analysed statistically with paired Student’s t tests.

Lumbar sympathetic stimulation (between L3 and L4 ganglia) (8V, 20-30Hz, 1ms pulse duration) had no effect on either EAS or IAS but was effective in eliciting a contraction of the anococcygeus muscle (16.4 ± 5.4 mN; n=5). Repetitive single twitch contractions of the EAS were elicited by stimulating the left inferior rectal nerve at 1Hz (3-6V, 1ms pulse duration). These EAS striated muscle twitches were superimposed on spontaneous oscillations of the IAS smooth muscle (ultra slow waves; amplitude: 10.7 ± 3.7 mN; frequency: 6 every ten minutes). The amplitude of striated contractions at the peak of the ultra slow wave was 7.0 ± 3.6 mN. As the smooth muscle relaxed, the amplitude of the striated twitch increased to 9.2 ± 4.5 mN which represents a 32.1 ± 8.8% rise (n=5, p<0.01). When the canal was decentralized by transecting both inferior rectal nerves (IRNs), this modulation of EAS force by underlying slow wave activity was still evident in all animals. The percentage increase following IRN transection was not significantly different to the values of the intact anal canal: 34.7 ± 27.9% (n=5, p=0.8).

Standard immunocytochemical methods were used to visualise motor end plate complexes (primary antibodies to neurolipin and synaptic vesicle 2 were combined with Alexa fluor-594 labelled α-bungarotoxin). The animals were anaesthetised with 5% isoflurane in oxygen (0.5/l/min). Once anaesthetised intraperitoneal gentamycin (6mg/kg) were transected 55 ± 30.5% (n=6) of endplates were denervated 5 days later.

In conclusion, the rat anal sphincters are not affected by sympathetic stimulation. When the smooth muscle of the IAS relaxes spontaneously neurally evoked EAS contractions increase in amplitude. This modulation of the EAS by slow waves in the IAS is unaffected by severing pudendal nerve motor fibres. As only half the motor endplate are denervated after bilateral IRN section these findings provide tentative evidence for a dual innervation of the rat EAS. The first of these is from the pudendal nerve via its inferior rectal branches and the source of the other may be the enteric nervous system or pelvic nerves.

This research was supported by the School of Medicine and Medical Sciences, University College Dublin.

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PC178

A murine model of slow transit constipation induced by outlet obstruction

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Background: The spontaneous colonic migrating motor complex (CMMC) is responsible for fecal pellet migration. Colonic elongation evokes the “occult reflex” that activates mechanosensitive descending interneurons that release nitric oxide to inhibit the neural circuitry underlying the CMMC (Heredia et al., 2010). It has been suggested that the “occult reflex” activated by colonic elongation may contribute to slow transit constipation (STC) in humans (Southwell, 2010). The aim of this study was to produce a non-pharmacological, phenotypically analogous and easily inducible model for STC. Methods: The maximal anal sphincter opening was physically reduced in anesthetized (isoflurane inhalation, 1.75%) C57 mice to 2.5 mm using a purse string suture. 4 days following suture placement, mice were either sacrificed (obstructed) or the suture removed (relieved). Following sacrifice the entire gastrointestinal tract was removed. Spontaneous fecal pellet transit and colonic motility were recorded using a CCD camera and isometric tension transducers respectively. Intracellular microelectrodes were used to record electrical activity of the circular muscle. Results: Obstructed mice compared to controls exhibited an elongated (control 49.7 ± 0.8mm; obstructed 66.3 ± 1.6mm; p<0.01, n=5) impacted large bowel with no discrete fecal pellets; a reduced daily output of fecal dry matter (control 0.40 ± 0.03g and obstructed 0.13 ± 0.01g; p<0.01, n=10); slowed transit of an artificial pellet along the emptied colon (control 0.68 ± 0.07mm/s and obstructed 0.28 ± 0.13mm/s; p<0.01, n=5), and abnormal or uncoupled CMMC propagation. As the obstructed colon slowly emptied it became hyperactive, generating more frequent CMMCs. The maximal anal sphincter opening was decreased in anesthetized (isoflurane inhalation, 1.75%) C57 mice to 2.5 mm using a purse string suture. 4 days following suture placement, mice were either sacrificed (obstructed) or the suture removed (relieved). Following sacrifice the entire gastrointestinal tract was removed. Spontaneous fecal pellet transit and colonic motility were recorded using a CCD camera and isometric tension transducers respectively. Intracellular microelectrodes were used to record electrical activity of the circular muscle. Results: Obstructed mice compared to controls exhibited an elongated (control 49.7 ± 0.8mm; obstructed 66.3 ± 1.6mm; p<0.01, n=5) impacted large bowel with no discrete fecal pellets; a reduced daily output of fecal dry matter (control 0.40 ± 0.03g and obstructed 0.13 ± 0.01g; p<0.01, n=10); slowed transit of an artificial pellet along the emptied colon (control 0.68 ± 0.07mm/s and obstructed 0.28 ± 0.13mm/s; p<0.01, n=5), and abnormal or uncoupled CMMC propagation. As the obstructed colon slowly emptied it became hyperactive, generating more frequent CMMCs. The colonic muscle in the obstructed colon was further depolarized (control -61.3 ± 4.1mV and obstructed -49.8 ± 6.2mV; p<0.01, n=5) owing to a lack of spontaneous inhibitory junction potentials. Colonic elongation applied to the empty obstructed colon produced a pronounced “occult reflex”. In 3 out of 10 animals we observed a jejunal volvulus (ischemic twisted section of bowel). In relieved animals (7 days post suture removal) there was no significant difference in colonic
size, fecal output, fecal transit speed, CMMC propagation, membrane potential, and spontaneous inhibitory junction potential occurrence from controls. Conclusions: These results illustrate parallels between surgically obstructed mice and outlet obstruction induced STC in humans: slowed fecal pellet transit, decreased fecal output and elongated large bowels. Interestingly, outlet obstruction appears to produce a hyperexcitable colon including an absence of tonic inhibition of the circular muscle. This hyperexcitability appears to be suppressed by the “occult reflex” triggered by colonic elongation. Heredia, D.J. Dickson, E.J. Bayguinov, P.O. Hennig, G.W. Smith, T.K., 2010. Colonic elongation inhibits pellet propulsion and migrating motor complexes in the murine large bowel. The Journal of Physiology 588(15), p. 2919-2943.


Support: NIH NIDDK R01 DK45713

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PC179

Calcium activity in submucosal neurons during the colonic migrating motor complex (CMMC) in the isolated murine large intestine

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Background: The CMMC is a spontaneous, rhythmic, neurally mediated motor pattern that is responsible for the propulsion of fecal pellets in mice (Heredia et al., 2010). The enteric nervous system consists of two ganglionated neural networks called the myenteric plexus, which lies between the longitudinal and circular muscle layers, and the submucosal plexus, which lies in the submucosa. The CMMC is generated by neurons within the myenteric plexus (Bayguinov et al., 2010), whereas neurons in the submucosal plexus regulate the secretion of water and electrolytes respectively. It is likely that these plexuses normally function as one system, since 3 of the 4 myenteric descending interneurons also project to the submucosal plexus, and sensory neurons in both plexuses communicate with one another (Furness, 2006). In this study, we have used Ca2+ imaging to examine whether submucosal neurons are activated during the CMMC. Methods: Mice (C57) were anaesthetized by inhalation of 5% isoflurane in 97% O2 + 3% CO2, and killed by cervical dislocation. The whole murine colon was removed and opened along the mesenteric border and pinned with the mucosa uppermost in a recording chamber perfused with oxygenated Krebs’ solution at 34°C. A section of the mucosa was peeled away to expose the submucosal ganglia, which were loaded with Fluo-4 (Bayguinov et al., 2010). CMMCs either occurred spontaneously or were evoked by mechanically stimulating the mucosa with a brush or by colonic elongation, both applied at the anal end of the large bowel (Bayguinov et al., 2010; Heredia et al., 2010). Results: Values are means ± S.E.M. A spontaneous or an evoked CMMC was indicated by a contraction (duration: 25.0 ± 4.4 s; n=5) of the tissue. The majority of neurons (~70%) in submucosal ganglia (7.3 ± 1.4 neurons; n=5) exhibited spontaneous Ca2+ transients (frequency = 0.12 ± 0.03 Hz; n=15). During an evoked CMMC, submucosal neurons responded in 2 different ways: a series of fast Ca2+ transients superimposed on a prolonged rise in Ca2+ (frequency = 0.6 ± 0.1 Hz, duration ≈ 14.4 ± 1.5 s; n=16) after a latency of 6.4 ± 1.4 s (measured from onset of contraction), and/or an increase in Ca2+ transients which began during the latter part of the CMMC (frequency = 0.6 ± 0.1 Hz, duration = 11.5 ± 2.7 s, latency 19.5 ± 2.1 s; n=9). Conclusion: Submucosal neurons are likely activated by myenteric neurons during the CMMC. Their different responses may represent their diverse functions (see Furness, 2006). However, it seems likely that there is an overall increase in secretion during fecal pellet propulsion.


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PC180

The rapid neural response to slow platform tilts in standing man

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Gurfinkel et al (1995) have previously described the reactions of standing human subjects to slow tilts of the support surface. Averaged records showed that a constant velocity platform tilt of 0.05°/s initially resulted in a relatively fast body inclination in the direction of the tilt. After a period of around 3-5 seconds, stronger resistance to body inclination began. Gurfinkel et al suggested the cause of this initial body deviation was due to mechanical yielding of the calf muscle/tendon/joint unit under conditions of support surface tilt. Neural intervention occurred only as the body slowed after the initial inclination. The recent discovery (Casadio et al., 2005; Loram & Lakie, 2002) that ankle stiffness is inadequate on its own to provide stable standing has led us to reinvestigate this behaviour.

Subjects (n = 5, 3 male) stood quietly with eyes closed on custom built footplates controlled by a servo motor which generated slow rotations in the sagittal plane around the axis of the ankle joint. Trials consisted of a baseline period lasting between 10 and 15 seconds, followed by a constant velocity (0.05°/s) toes-up, or toes-down tilt lasting 10 seconds. 20 toes-down, and 20 toes-up tilts were completed for each subject. We combined high quality ultrasound imaging of the soleus and gastrocnemius muscles with a novel automatic tracking technology to provide a continuous measure of muscle length change for the duration of the initial response. Ankle and platform angles were measured by laser retroreflective rangefinders and surface EMG recorded from the soleus and gastrocnemius muscles.

Our averaged results of a toes-down tilt of the support surface (Fig 1. top) confirm that the initial rapid body deviation, as measured by ankle angle (Fig 1. middle), lasted around 3 seconds. This was accompanied by a shortening of the soleus and gastrocnemius muscles (Fig 1. bottom). Conversely, toes-up tilts resulted in a corresponding backward body inclination accompanied by a lengthening of the calf muscles. Changes
in calf muscle EMG were observable within 500 ms of the start of the ramp. Mechanically, the calf muscles would be expected to lengthen as the body leant forwards, and shorten as the body leant backwards. Out results show the opposite demonstrating that neural rather than purely mechanical processes are involved in the initial response to slow support surface tilts.


TO is supported by EPSRC

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PC181

Selective inhibition of GluN2A-containing NMDARs by an antagonist acting at the GluN1 subunit

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The ability to discriminate between subtypes of N-methyl-D-aspartate receptors (NMDARs) is highly desirable in order to determine their various contributions to physiological and pathophysiological processes in the CNS. In the forebrain GluN2A and GluN2B are the most abundant GluN2 NMDAR subunits but a selective antagonist that inhibits GluN2A-containing while sparing GluN2B-containing NMDARs is lacking (Frizelle et al. 2006). A recent report by Bettini et al. (2010) indicated that N-(cyclohexylmethyl)-2-[(5-[phenylmethyl]amino)-1,3,4-thiadiazol-2-yl]thio)acetamide (referred to as Compound 13 in their study and abbreviated here to N-CPTTA) showed selective antagonism of recombinant GluN1/GluN2A NMDARs while having little action at GluN1/GluN2B NMDARs. We have characterized of the antagonist action of N-CPTTA in both recombinant and native NMDARs. Our data indicate that N-CPTTA is able, under certain conditions, to selectively block GluN2A-containing NMDARs while displaying no antagonism at GluN2B-containing NMDARs but does so by acting at the GluN1 NMDAR subunit.

Two-electrode voltage-clamp recordings were made from Xenopus laevis oocytes expressing either GluN1/GluN2A or GluN1/GluN2B rat recombinant NMDARs. For GluN1/GluN2A NMDARs antagonism by N-CPTTA was dependent on the glycine concentration used in our external recording solution and was independent of the glutamate concentration. Concentrations of N-CPTTA that produced 50% inhibition of glutamate (100 μM) evoked currents when the external glycine concentration was 150 nM, 1.5 μM and 15 μM were 0.55 ± 0.05, 3.5 ± 0.3 and 39.7 ± 3.4 μM respectively (n = 7, 7, 6). Antagonism by N-CPTTA was surmountable and competitive with Schild analysis giving an estimated Kᵦ value of 2 μM (n = 5). For GluN1/GluN2B NMDARs N-CPTTA (10 μM) only produced modest antagonism (5.5 ± 3.4%; n = 6) of agonist evoked currents even when the glycine concentration was low (75 nM) i.e. 10% of its EC₅₀ value at GluN2B-containing NMDARs (Chen et al. 2008). The prototypical competitive glycine-site antagonist, 5,7 dichlorokynurenic acid, did not show selective antagonism and gave comparable IC₅₀ values at GluN2A- and GluN2B-containing NMDARs when equi-potent glycine concentrations were used.

Additionally we examined the effects of N-CPTTA acting at NMDARs expressed in rat cultured cortical neurones at a stage where NMDARs predominantly contain GluN2B subunits. Our data indicate that N-CPTTA produces little block of NMDA-evoked currents whereas ifenprodil, a selective blocker of GluN2B-containing NMDARs when equi-potent glycine concentrations were used.

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PC182

The activation mechanism of rat α3 homomeric Glycine receptors

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The α3 subunit of the glycine receptor (GlyR) is found in the spinal cord and hippocampus, where it is thought to be involved in pain sensitivity and in some forms of epilepsy. Despite the physiological relevance of α3-containing channels, there are no studies on kinetic properties of the α3 homomeric receptor. Here we investigated these properties by expressing rat homomeric α3 GlyR (GenBank accession number AJ310838) in HEK293 cells. Cell-attached single channel recordings were obtained at a range of glycine concentrations (50 – 10000 μM). Macroscopic synaptic-like glycine-evoked currents were obtained by rapid applications of brief pulses of saturating concentrations.
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resting state value of 1340 et al.
was very high both with three and four glycine molecules except that with α3 channels we can see that a fourth molecule of glycine can bind to the channel, something that we could not clarify for the α1 or α11 channel. Open probability was very high both with three and four glycine molecules bound. α11 and α3 GlyRs are similar in their microscopic affinity for glycine and in the efficiency with which the channel flips and then opens. In particular, the opening rate constant was 150,000 ± 20,000 s⁻¹ and the overall efficacy was 62 ± 5 (n = 3 sets; cf. 129,000 ± 1 and 20 for α11 heteromers; Burzomato et al., 2004). Again, the microscopic affinity for the agonist glycine was seen to increase as the channel activated, from a resting state value of 1340 ± 200 μM to 180 ± 40 μM (n = 3 sets) for the partially activated flip state.

This mechanism and rate constants were also found to describe well the time-course of synaptic-like glycine currents, predicting a decay time constant of 12 ± 4 ms (n = 3 sets), cf. experimental value of 8 ± 3 ms (n = 5 patches; randomisation test, p = 0.20).


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PC183

Structural re-arrangement of the BK channel RCK1-RCK2 linker investigated using fluorescence lifetime imaging microscopy

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Alternative splicing generates considerable functional diversity in large conductance calcium- and voltage-activated potassium (BK) channels. For example, inclusion of the STREX (STress-activated EKx) insert into the cytosolic linker between the two regulator of potassium conductance (RCK) domains of the channel generates a cysteine rich domain (CRD) that confers intrinsic hypoxia sensitivity to the channel (McCartney et al., 2005), as well as sites for regulation by phosphorylation and palmitoylation (Tian et al., 2008).

The RCK1-RCK2 linker region is considered unstructured yet has clear impact on BK channel function and so we have developed YFP-mCFP fluorescent fusion proteins of the CRD and exploit these to examine conformational rearrangements in the CRD in response to hypoxia using fluorescence lifetime imaging microscopy (FLIM). YFP-mCFP fusion proteins of the CRD encompassing the STREX insert are targeted to the plasma membrane of HEK293 cells. Exposure of intact cells expressing the construct to acute hypoxia (10 minute exposure at <5% oxygen) resulted in a significant shift in fluorescence lifetime distribution (n=9). This suggests that hypoxia induces a conformational rearrangement in the RCK1-RCK2 linker. Site directed mutagenesis of the cysteine residues within STREX, which we had previously shown to confer functional hypoxia sensitivity to the channel, abolished the hypoxia-induced shift in lifetimes (n=7). Pre-treatment with either oxidizing or reducing agents did not block the hypoxia-induced shift in fluorescence lifetimes (n=6). The effects of cysteine mutagenesis and REDOX manipulation on hypoxia sensitivity were recapitulated in patch clamp assays of channel activity (n=3 to 9 for each construct) supporting a role for hypoxia-induced shifts in CRD conformation with changes in channel function.

These findings suggest that FLIM, in conjunction with patch clamp electrophysiology, will be an important approach to monitor conformational rearrangements and investigate the structure-function relationship of the important unstructured linker region between the channel RCK domains.


This work was supported by the Wellcome Trust

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC184

Recovery of Precise Upper Limb Force Production Enables Better Hand Position Control Early After a Stroke

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The recovery of upper limb force production is highly variable across patients early after stroke and many have long-term arm impairment and disability. Arm strength is often measured by maximal voluntary isometric contractions (MVC), which engage large muscle masses that do not mimic activities of daily living (ADLs) and are accompanied by little attention to precision of force production and position control. The main purpose of this study was to demonstrate that position control using precise low-level force production and its modulation against direction-dependent force perturbations recovers in a way that is more correlated to functional clinical outcome measures than MVCs.

Consecutive patients were recruited from an inpatient stroke unit (n=10). Each patient was in early recovery (<7 weeks post-lesion) from their first ever stroke. Each patient was instructed to maintain a central "hold" position task with a hand-held joystick, whilst a robot device imposed a time-varying planar force perturbation in each of 8 directions (IMT2 robot device; Boston, MA, USA). Evaluations of precise force production (Newtons; N) and position error (meters; m) during the task, as well as elbow flexion MVC and clinical outcome measures of motor
improvement (Fugl-Meyer score; FM) were performed before (PRE), once a week and then after (POST) 5 weeks of recovery. Changes in mean peak force produced by the patient to counteract the robot-induced force perturbation and mean peak planar X-Y position error of the joystick (averaged across the 8 force directions), MVC (elbow flexion measured with the robot device) and FM score were compared across the recovery period using repeated measures ANOVA and post-hoc paired Student’s T-tests adjusted for multiple comparisons. Significant differences in PRE vs. POST values (mean ± SEM) are reported at the p < 0.0125 level.

Mean peak force production improved during recovery after stroke (17 ± 2 vs. 26 ± 2 N; PRE vs. POST; p = 0.002), whereas elbow flexion MVC did not (25 ± 3 vs. 23 ± 5 N; p = 0.71). As a result, mean peak position error decreased during recovery (0.08 ± 0.01 vs. 0.06 ± 0.01 m; p = 0.0001). Mean peak position error (y) was inversely and linearly correlated to FM score (x) during recovery (y = 60 ± 8 - 409 ± 104 x; r = -0.60; p = 0.0005).

This study demonstrated that improvement in precise force production can operate as a mechanism enabling better motor behaviour such as hand position control and suggests that low-level force production and its modulation could be a target for future neurorehabilitation strategies during early recovery from a stroke. Secondly, robot-based kinematic measures were significantly correlated with clinical outcome and may act as surrogate indices of recovery.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC185**

**Effects of End Stage Chronic Renal Failure on L-arginine-nitric oxide pathway and urea cycle in red blood cells**

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Chronic renal failure (CRF) is characterized by the presence of endothelial dysfunction, associated with increased bioavailability of nitric oxide (NO) in blood cells (1). L-arginine is a cationic amino acid that is converted into NO by a family of enzymes referred as NO synthase (NOS). It is also converted into L-ornithine and urea by arginase and both enzymes compete for the same substrate (2). Recent evidence has shown that red blood cells (RBC) produce and release NO, which regulates RBC deformability and promotes vasodilatation (3). The aim of the present study was to investigate the effects of CRF on the L-arginine-NO pathway, the arginase pathway in RBC, and RBC osmotic fragility (OF) as a measure of membrane instability. Were included In this study 13 CRF patients under haemodialysis and 12 healthy controls. L-arginine influx was determined by measuring the uptake of L-[3H]-arginine (5-100 μM). N-ethylmaleimide was used to isolate transport system y+L. Basal NOS activity was measured by the conversion of L-[3H]-arginine into L-[3H]-citrulline. Expression of arginase I and II was accessed by Western Blotting. Arginase activity was analyzed in RBC lysate through the conversion of [C14]-L-arginine into [C14]-urea. Of was assessed by the incubation of RBC with different concentrations of NaCl. The present study was approved by the Pedro Ernesto Hospital Ethical Committee (process 451-CEP/HEUPE), and informed consent was obtained from each participant. Values are means ± S.E.M. compared by the Student’s t-test. L-arginine influx (μmol/L/cells/h) was increased in RBC from CRF patients via system y+L (1908 ± 364 vs. 638 ± 109, p<0.05) and y+L (771 ± 230 vs. 221 ± 64, p<0.05), however, no difference was detected in NOS activity (IRC: 0.031 ± 0.001 vs. 0.037 ± 0.005 nmol/108 cells, controls). RBC expressed only arginase I and CRF patients showed an over-expression of this enzyme (5 replicates of each group) associated with increased enzyme activity (IRC: 34.4 ± 5 vs. 21 ± 2 nmol urea/mg protein/ 2h controls, p<0.05). RBC from CRF patients showed increased OF at 0.7 (IRC: 18.9 ± 5 vs. 5.3 ± 2 % haemolysis controls, p<0.05) and 0.65 % NaCl (IRC: 34.1 ± 12 vs. 7.5 ± 3 % haemolysis controls, p<0.05). Thus the present study showed that L-arginine influx is increased in RBC from CRF patients, which could be explained in part by alterations in the plasmatic membrane of these cells. However, in these patients the increased uptake of L-arginine is probably shifted towards the urea cycle, with activation of arginase I and higher levels of this enzyme in RBC. Even though there is an upregulation of the arginase pathway, NOS activity remained within normal levels. These data suggest that in RBC, L-arginine influx and arginase I are key factors involved in the alterations of erythrocytes detected in CRF.

Brunini TM, Yaoqob MM, Novaes Malagris LE, Ellory JC, Mann GE, Mendes Ribeiro AC. Increased nitric oxide synthesis in uraemic platelets is dependent on L-arginine transport via system y+L. Pflugers Arch. 2003;445:547-50.


Financial Support: FAPERJ, CNPq, CAPES.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC186**

**The lipoxin A4 increases ASL height in normal and cystic fibrosis bronchial epithelium**

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Mutations of the Cystic Fibrosis (CF) gene result in defective Cl- secretion and Na+ hyperabsorption. This contributes to a reduction of the airway surface liquid layer (ASL) height and impairs mucociliary clearance, thus promoting bacterial colonization and chronic inflammation. Identification of agents that promote hydration of the ASL is likely to be of therapeutic benefit to patients with CF.

Lipoxins are bioactive lipid mediator produced at inflamma
tory sites from the interaction of lipoygenase activities of several cell types including neutrophils, platelets and epithelium. This lipid mediator is one member of the newly identify mol-
ecules playing a role in ending/resolving the inflammatory process by modulating neutrophilic inflammation, clearing
apoptotic PMN and inhibiting pro-inflammatory cytokines production. The levels of LXA4 have been reported to be decreased in the airways of patients with CF.

We have discovered a pro-secretory effect of the endogenous LXA4 in human bronchial epithelium. Human bronchus epithelial (HBE) cell primary cultures and CF(CuFi-1) and non-CF(NuLi-1) bronchial epithelial cell lines were grown under an air-liquid interface to well-differentiated epithelia. LXA4 (1 nM) treatment significantly increased ASL height in non-CF and CF HBE. This effect was sustained over 24 hours in the CF HBE and was inhibited by Boc-2, the antagonist of the ALX/FPR2 receptor that we found to be expressed in the apical membrane of HBE. We investigated the contribution of Na+ absorption (via ENaC) and of Cl− secretion in the ASL height regulation by LXA4. LXA4 pre-treatment reduced the amiloride-sensitive short-circuit current in CuFi-1 epithelium indicating that LXA4 inhibits ENaC activity. LXA4 and amiloride produced additive stimulating effects on the ASL height. LXA4 increased the whole-cell currents of non-CF and CF HBE and this effect was inhibited by BAPTA-AM (chelator of intracellular Ca2+) and NPPB (non-selective inhibitor of Ca2+-activated Cl- channels) but not by the CFTRinh-172 (CFTR inhibitor). Bumetanide abolished the ASL height increase induced by LXA4. In addition, LXA4 stimulated an apical ATP release. Hexokinase (ATP hydrolysis), reactive-blue-2 (P2Y purinoreceptor antagonist) and NF340 (P2Y 11 purinoreceptor antagonist) abolished the LXA4 effect on ASL height.

Taken together, our results provide evidence for a novel effect of LXA4 involving the FPR2 receptor in the apical membrane, luminal ATP secretion and P2Y receptor(s) activation inhibition of Na+ absorption and stimulation of Cl− secretion in CF and non-CF epithelia to finally increase ASL height. These novel pro-resolving effects of LXA4 reveal a cross-talk between an endogenous anti-inflammatory mediator decrease and the ion transport defect in CF and open up a new therapeutic avenue in the treatment of CF.

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PC187

Secretion by the human airway epithelial cell line Calu-3 depends on basolateral chloride loading by AE2

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The anion exchanger type 2 (AE2) carries out Na+-independent Cl−/HCO3− exchange in non-epithelial cells and at the basolateral membrane of many epithelia, however its role in transport across airway epithelial cells is poorly understood. We have identified the splice variants AE2a and AE2b2 in the Calu-3 cell line, a commonly used model for human airway submucosal gland serous cells. To assess their physiological role, we targeted both variants using lentivirus-mediated RNA interference to generate a stable AE2 knock-down Calu-3 cell line. The levels of LXA4 have been reported to be decreased in the airways of patients with CF.

We have discovered a pro-secretory effect of the endogenous LXA4 in human bronchial epithelium. Human bronchus epithelial (HBE) cell primary cultures and CF(CuFi-1) and non-CF(NuLi-1) bronchial epithelial cell lines were grown under an air-liquid interface to well-differentiated epithelia. LXA4 (1 nM) treatment significantly increased ASL height in non-CF and CF HBE. This effect was sustained over 24 hours in the CF HBE and was inhibited by Boc-2, the antagonist of the ALX/FPR2 receptor that we found to be expressed in the apical membrane of HBE. We investigated the contribution of Na+ absorption (via ENaC) and of Cl− secretion in the ASL height regulation by LXA4. LXA4 pre-treatment reduced the amiloride-sensitive short-circuit current in CuFi-1 epithelium indicating that LXA4 inhibits ENaC activity. LXA4 and amiloride produced additive stimulating effects on the ASL height. LXA4 increased the whole-cell currents of non-CF and CF HBE and this effect was inhibited by BAPTA-AM (chelator of intracellular Ca2+) and NPPB (non-selective inhibitor of Ca2+-activated Cl- channels) but not by the CFTRinh-172 (CFTR inhibitor). Bumetanide abolished the ASL height increase induced by LXA4. In addition, LXA4 stimulated an apical ATP release. Hexokinase (ATP hydrolysis), reactive-blue-2 (P2Y purinoreceptor antagonist) and NF340 (P2Y 11 purinoreceptor antagonist) abolished the LXA4 effect on ASL height.

Taken together, our results provide evidence for a novel effect of LXA4 involving the FPR2 receptor in the apical membrane, luminal ATP secretion and P2Y receptor(s) activation inhibition of Na+ absorption and stimulation of Cl− secretion in CF and non-CF epithelia to finally increase ASL height. These novel pro-resolving effects of LXA4 reveal a cross-talk between an endogenous anti-inflammatory mediator decrease and the ion transport defect in CF and open up a new therapeutic avenue in the treatment of CF.

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PC188

Cyclic AMP-induced phosphorylation of endogenous Nedd-4/2 and surface abundance of epithelial channel subunits (α, β- and γ-ENaC) in H441 human airway epithelial cells

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H441 human distal airway epithelial cells expresses an endogenous Na+ conductance identical to that associated with α, β- and γ-ENaC co-expression. This conductance is quiescent in hormone-deprived cells but can be activated by dexamethasone, and this glucocorticoid-induced Na+ conductance (GNa) is further augmented by brief (20 min) activation of protein kinase A (PKA) (Clunes et al., 2004). Glucocorticoids seem to control ENaC via serum and glucocorticoid-inducible kinase 1 (SGK1), a kinase (Lang et al., 2006) that phosphorylates residues (Ser221, Thr246, Ser327) in Nedd-4/2, ubiquitin ligase that normally targets ENaC for internalization / degradation. By phosphorylating Nedd4/2, SGK1 seems to prevent this interaction with ENaC thus allowing the channels to remain in the membrane leading to a rise in GNa and stimulation of Na+ transport. Moreover, since PKA can also phosphorylate Nedd4/2-Ser221, -Thr246 and -Ser327, it has been suggested that CAMP-coupled agonists also augment GNa by increasing the surface abundance of α-, β- and γ-ENaC (Snyder et al., 2004). We have therefore explored the effects of PKA activation upon the phosphorylation of endogenous Nedd-4/2 and the surface abundance of α-, β- and γ-ENaC in H441 cells.

Dexamethasone (0.2 μM, 24 h) had little effect upon Nedd-4/2 phosphorylation, but did evoke phosphorylation of this protein after only 3 h stimulation (n = 3, not shown), a finding...
which accords with data showing that dexamethasone activates SGK1 transiently (Ismail et al. 2011). The cAMP-elevating drugs evoked phosphorylation of the cAMP response element binding protein Ser133 confirming activation of PKA, and this response was accompanied by an increase in the abundance of Ser221, Thr246, and Ser327-phosphorylated Nedd-4/2 both in hormone-deprived and dexamethasone-stimulated cells. Interestingly, this response was always accompanied by a marked increase in the overall abundance of Nedd-4/2 (Fig. 1).

Dexamethasone (24 h) increased the surface abundance of α-ENaC with no such effect upon β- or γ-ENaC (see also Ismail et al. 2011). Activating PKA independently of dexamethasone (n = 4) mimicked the effects of glucocorticoid stimulation by increasing the surface amount of α-ENaC with no effect on the β- or γ-subunits (Fig. 4B). In 5 out of 11 experiments, exposing dexamethasone-stimulated cells to cAMP did not affect the surface abundance of α-, β- or γ-ENaC (Fig. 1B), but increased surface expression of β- and γ-ENaC, but not α-ENaC, was seen in 6 instances. Whilst it clearly phosphorylates Nedd-4/2 (Fig 1A) the PKA-induced increases in Gmax (Clunes et al. 2004) do not necessarily correlate with changes to the surface expression of ENaC subunits.

Fig. 1. (A) Confluent cells on 10 cm Petri dishes were lysed in the presence of protease and phosphatase inhibitors so that Nedd-4/2 could be immunopurified from 1 mg aliquots of extracted protein and subject to Western analysis using antibodies against the Ser221, Thr246- and Ser327-phosphorylated proteins and total forms of this protein. (B) Abundance of α-, β- and γ-ENaC in surface-exposed protein fraction isolated by biotinylation/streptavidin-binding. Dex: 0.2 μM dexamethasone for ~24 h; cAMP: 10 μM forskolin, 100 μM isobutylmethylxanthine, 1 mM N6,2’-O-dibutyryl adenosine 3’5’-cyclic monophosphate for the final 20 min of this incubation period.

Ismail NAS et al. (2011). Proc Physiol Soc (Durham meeting)

Supported by a grant from the Wellcome Trust and a studentship from the Government of Malaysia

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PC189

The dual role of CO₂/HCO₃⁻ buffer in the regulation of intracellular pH of three-dimensional, multi-cellular tumour growths

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Intracellular pH (pHᵢ), a major modulator of cell function, is regulated by acid/base transport across membranes. Excess intracellular H⁺-ions (e.g. produced by respiration) are extruded by transporters such as Na⁺/H⁺ exchange, or neutralized by HCO₃⁻-taken up by carriers such as Na⁺/H⁺-HCO₃⁻ cotransport. Using fluorescence pH-imaging, we show that cancer-derived cell-lines (colorectal HCT116 and HT29, breast MDA-MB-468, pancreatic MiaPaca2, cervical HeLa) extrude acid by H⁺-efflux and HCO₃⁻-influx, largely sensitive to 30 μM dimethylamiloride (DMSO) and 100 μM 4,4’-diisothiocyanatostilbene-2,2’-disulfonate (DIDS), respectively. The magnitude of HCO₃⁻-influx was comparable among the cell-lines, and may represent a constitutive element of tumor pHᵢ regulation (3.0±0.4SEM to 4.3±0.4 mM/min at pHᵢ=6.7). In contrast, H⁺-efflux varied considerably (MDA-MB-468>HCT116>HT29>MiaPaca2> HeLa; from 7.0±0.4SEM to 0.7±0.2SEM mM/min at pHᵢ=6.7). When HCO₃⁻-flux was pharmacologically inhibited with DIDS, acid-extrusion in multi-cellular HT29 and HCT116 spheroids (radii 130-200 μm; ~10,000-50,000 cells) was highly non-uniform and produced low pHᵢ at the core (see Figure). With depth, acid-extrusion became relative more DIDS-sensitive because the low extracellular pH at the spheroid core inhibits H⁺-flux more than HCO₃⁻-flux. HCO₃⁻-flux inhibition also decelerated HCT116 spheroid growth. In the absence of CO₂/HCO₃⁻, acid-extrusion by H⁺-flux in HCT116 and MDA-MB-468 spheroids became highly non-uniform and inadequate at the core (see Figure). This is because H⁺-transporters require extracellular pH-buffers, like CO₂/HCO₃⁻, to overcome low H⁺-ion mobility and chaperone H⁺-ions away from cells. The effect of 5% CO₂/22 mM HCO₃⁻ was mimicked by 30 mM Hepes (an artificial buffer system) but reduced when carbonic anhydrase activity (which normally keeps CO₂/HCO₃⁻ at equilibrium) was inhibited with acetazolamide (100 μM; see Figure). CO₂/HCO₃⁻ exerts a dual effect: as substrate for membrane-bound H⁺-transporters, and as a mobile buffer for facilitating extracellular diffusion of H⁺-ions extruded from cells. These processes can be augmented by carbonic anhydrase activity. We conclude that CO₂/HCO₃⁻ is important for maintaining uniformly alkaline pHᵢ in small, non-vascularized tumour-growth and may be important for cancer disease progression.
Orai3 contributes to breast cancer development through c-Myc oncogene pathway activation

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Considerable advances in the molecular understanding of human breast cancer (BC) have emerged during the last decades. However, BC remains, at the dawn of the third millennium, one major public health problem. The main goal of scientists is to develop more efficient drugs targeting proteins involved in regulation of cell proliferation and/or apoptosis. In this context, one of the most promising signaling pathways to target for cancer therapy is the c-Myc pathway (Vita and Henriksson, 2006). In BC, several studies show that 50 to 100% of BC cases present increased levels of c-Myc proteins (Liao and Dickson, 2000). The importance of c-Myc as a potential therapeutic target is due to its involvement as a key actor in G1 phase progression and G1/S transition.

The newly discovered calcium channels Orai are known for their pathophysiological roles (Feske, 2010). Nevertheless, their expression and role in BC has only recently emerged (Yang et al., 2008; Faouzi et al., 2011). We have reported that Orai3 channels are overexpressed in more than 70% of BC biopsies (all tissue samples obtained from surgery, and both tumour and normal tissues were taken from the same operative specimen). We have also shown that these channels are involved in proliferation, cell cycle progression and survival of BC cells by regulating the G1 phase and G1/S transition regulator proteins (Faouzi et al., 2011). The importance of these channels is highlighted by the fact that their described role in BC cells seems to be specific to cancer status, since their downregulation does affect neither cell proliferation, nor cell survival in normal breast cells.

On the basis of the whole reported data, we hypothesized that Orai3 channels could be upstream regulators of the c-Myc pathway. Thus, we investigated the role of c-Myc in the Orai3-induced proliferation and cell cycle progression in BC cells compared to normal breast cells. Our results showed that Orai3 silencing reduced both c-Myc expression and activity levels in BC cells only. Simultaneous downregulation of both Orai3 channels and c-Myc protein had no additive or synergic effects on either BC cell proliferation or cell survival. One of the known pathways regulating c-Myc protein expression and activity is the MAP Kinase pathway. We therefore investigated the effect of Orai3 downregulation on ERK1/2 phosphorylation. Our results showed that the phosphorylation level of ERK1/2 significantly decreased when Orai3 channels were downregulated.

To conclude, our results suggest that Orai3 channels are one of the upstream regulators of the oncogenic c-Myc pathway that constitute key players in BC development.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Deoxygenation-induced phosphatidylserine exposure in human red blood cells from sickle cell patients

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The aminophospholipid phosphatidylserine (PS) is usually confined to the inner leaflet of red blood cells (RBCs). This is important because externalisation can result in thrombus formation and removal of RBCs. Externally PS occurs in a small, but variable, percentage of RBCs from sickle cell disease (SCD) patients and may participate in the chronic anaemia and acute ischaemic complications, characteristic of SCD (1). Exposure increases on deoxygenation. Whilst this appears to be stimulated by elevated extracellular Ca2+, the mechanism remains unclear (2). It is hypothesised that the deoxygenation-induced cation channel (P_{sickle}) of SCD patient RBCs is involved. Routine discarded blood samples were obtained from HbSS and HbSC SCD patients, using EDTA as anticoagulant. RBCs were washed and incubated in saline comprising (in mM): 4 KCl, 145 NaCl, 10 HEPES, 10 inosine, 0.15 MgCl2 with 1.1 CaCl2 unless otherwise indicated. Suspensions of RBCs (5% haematocrit, Hct) were deoxygenated (60min or less) using Eschweiler tonometers before diluting into test tubes pre-equilibrated with N2 (final Hct 0.5%) in the absence or presence of various potential inhibitors of PS exposure (10, 50 and 100μM). In some experiments, deoxygenated RBCs were preloaded with the Ca2+ chelator MAPTAM, before adding to test tubes. PS exposure was assessed by incubation with FITC-lactadherin (16nM, 10^6 RBCs, Haematologic Technologies Inc.) in the presence of vanadate (1mM) and analysed by flow cytometry (3). Deoxygenated RBCs with 1.1mM Ca2+ showed PS exposure of 3.8±0.3% (means±SEM, n=17), rising to 5.2±0.4 and 7.2±0.6% after 30 and 60min, respectively. In the absence of Ca2+, PS exposure was reduced by 65.2±24.3% (n=10, p<0.02) after 60min of deoxygenation. Pre-incubation with MAPTAM inhibited PS exposure at 60min by 58.4±27.9%, n=4, p<0.02 (Figure 1). Partial inhibitors of P_{sickle} DIDS, SITS and dipyridamole showed inhibition by 86.5±28.6, 91.9±25.4 and 50.4±25.0% (n=5, p<0.02), respectively. Inhibitors of the non-selective cation channel, EIPA, or of the peripheral benzodiazepine receptors, PK11195, had no effect. Data are shown for HbSS patients, but RBCs from HbSC genotype behaved similarly. Glutamate receptor agonists NMDA and homocysteine (HC) had little effect on PS exposure after 60min incubation in oxygenated RBCs being 2.5±0.6% (n=2) in controls and 2.3±0.2 and 2.6±0.3% with NMDA and HC (100μM), respectively. Findings show that Ca2+ was required for deoxygenation-induced PS exposure in sickle cells. The sickling shape change alone was insufficient. Intracellular Ca2+ chelation reduced the effect. Inhibitor data are consistent with Ca2+ entry via the P_{sickle} pathway with no evidence for involvement of other putative RBC cation channels. In future work, the link between Ca2+ and PS exposure will be pursued.

Figure 1: Membrane transport in blood cells from HbSC patients at different oxygen tensions.

Symbols represent means±S.E.M (n=3). Full deoxygenation (0mmHg vs. 100mmHg(normoxia)) significantly changed all components of K+ influx and cell sickling (p<0.02, Student’s t-test).


We thank the Medical Research Council for financial support.

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Pancreatic duct epithelium produces a HCO₃⁻-rich (~140 mM) isotonic fluid secretion, which conveys the protein-rich acinar secretion to the duodenum. It is generally thought that Na⁺-HCO₃⁻ cotransport (NBC1) provides a major route for HCO₃⁻ accumulation across the basolateral membrane. However, the role of the electroneutral Cl⁻-HCO₃⁻ exchange (AE2) at the basolateral membrane is not well understood.

To investigate the role of AE2, we have constructed two simulation models of the pancreatic duct epithelial cell using MATLAB/Simulink: a luminal-perfusion model and a fluid-secreting model. When AE2 was at its basal level, the secreted [HCO₃⁻]L was predicted to be ~85 mM. When AE2 activity was reduced to 50%, 10%, or 5%, [HCO₃⁻]L increased to ~102 mM, ~132 mM, and ~140 mM respectively (pHc was 7.30, 7.35, and 7.36 and apical membrane potential was -55 mV, -59 mV, and -60 mV). In the latter case, the rate of steady-state fluid secretion was ~1.2 nl min⁻¹ mm⁻² (per unit area of apical membrane), which is consistent with experimental data from guinea-pig pancreatic duct (Ishiguro et al., 1998). These results suggest that inhibition of AE2 is a prerequisite for HCO₃⁻-rich fluid secretion by pancreatic duct.


This work was supported by grants from the Japanese Society for the Promotion of Science and the Research Committee of Intractable Pancreatic Diseases provided by the Ministry of Health, Labor, and Welfare of Japan.

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Deoxygenation-induced Mg²⁺ currents in red blood cells from sickle cell patients

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Sickle cell disease (SCD) patients show chronic anaemia and acute ischaemic episodes. Their red blood cells (RBCs) have increased ion permeability, particularly when deoxygenated (1). This abnormality contributes to pathogenesis by causing solute loss and RBC shrinkage. Consequent elevation of [HbS] markedly encourages its polymerisation, RBC sickling and cation permeability of RBCs is therefore important in pathogenesis. Deoxygenation is also associated with activation of an as yet unidentified cation channel (2). Mg²⁺ depletion may increase activity of the KCl cotransporter which mediates coupled KCl efflux. Abnormal divalent cation permeability of RBCs is therefore important in pathogenesis. Deoxygenation is also associated with activation of an as yet unidentified cation channel (2). Mg²⁺ depletion may increase activity of the KCl cotransporter which mediates coupled KCl efflux. Abnormal divalent cation permeability of RBCs is therefore important in pathogenesis.
glucose 5, pH 7.4. Membrane conductance was ascertained using whole-cell patch clamp, as described previously (3), in fully oxygenated (O2 20%) or deoxygenated (O2 <1%) conditions. Pipette and bath solutions contained Mg2+ as the predominant cation (82mM). Flow perfusion was used to alter bath solutions and oxygenation. In some experiments, Gd3+ or streptomycin, both promiscuous stretch-activated channel blockers, were added to the bath. In symmetrical Mg2+ solutions (Fig 1), RBCs showed considerable Mg2+ conductance which increased on deoxygenation: at -90mV, currents were -79.3±23.0 and -304.2±68.8pA (n=10) at high and low O2, respectively (p<0.05). Currents were similar in magnitude to those recorded in symmetrical Na+ or K+ solutions. They were markedly inhibited by Gd3+ and much reduced by streptomycin (Fig 1): at -90mV, with Gd3+ (50μM) the current fell to -35.2±15.9pA (n=4) and with streptomycin (100μM) the current was -92.4±32.4pA (n=5), both p<0.05 cf no inhibitor. Similar results were obtained in Ca2+ solutions. Findings indicate elevated permeability of sickle cells to Mg2+ and Ca2+. Currents appear to be the electrical manifestation of P_sickle via stretch-activated channels, with elevated activity at high and low O2, respectively (p<0.05). Currents were similar inhibitors amenable to clinical use.

Patients with SCD have chronic alterations in Mg2+ and Ca2+ homeostasis observed in RBCs from SCD polymers. These electrical pathways could be involved in the following membrane distortion by deoxygenation-induced HbS polymerisation (1). Patients on intensive care with elevated ASL glucose are more likely to have respiratory infection, particularly with methicillin-resistant Staphylococcus aureus (MRSA; 2). The aim of this study was to investigate the relationship between mucosal glucose concentration, glucose in the ASL and bacterial growth using an in vivo model of human airway epithelium. H441 epithelial cells were grown on transwell inserts for 10 days, at air-liquid interface, to form polarised monolayers. Cells were washed and bathed in a HCO3 buffered Krebs solution to remove antibiotics and growth supplements which could effect bacterial growth. The glucose concentration of the mucosal Krebs solution was set at 10, 20 or 40mM. For ASL glucose analysis, cells were incubated for 1 hour with the different glucose concentrations. 150μl of solution containing 0.67μg ml⁻¹ FITC-dextran was used to wash the surface of the transwells. Glucose concentration of the washes was quantified using a fluorometer. ASL volume was calculated by fluid displacement. For co-culture studies, Staph aureus (8325-4) were grown overnight in RPMI medium to produce a culture of ~10⁶ colony forming units (CFU) ml⁻¹. 50μl of 10⁷ CFU ml⁻¹ in glucose-free RPMI was applied to the luminal surface of H441 monolayers. The H441-Staph. aureus co-culture was incubated for 7 hours at 37°C with 5% CO2. Transepithelial electrical resistance (TEER) of the monolayers was measured using a voltohmmeter (WPI). The co-culture was then scraped from the transwells and CFU's were determined using the Miles-Misra method. Calculated ASL glucose was less than mucosal glucose concentration but increased from 0.5±0.2mM to 1.5±0.2 and 6.5±1.3mM as basolateral glucose concentration was raised from 10mM to 20mM and 40mM, respectively (P<0.05; n=3-5). The TEER of H441 monolayers was significantly reduced following the addition of Staph. aureus (248±44 Ω cm²), compared to control monolayers (498±104 Ω cm²; P<0.05; n=5). Altering mucosal glucose had no effect on H441 TEER. Luminal Staph. aureus growth directly correlated with mucosal glucose concentration, increasing from 8.6±8.1x10⁷ to 1.5±0.8x10⁸ CFU ml⁻¹ and 3.4±1.4x10⁸ CFU ml⁻¹ upon elevation of mucosal glucose from 10mM to 20mM and 40mM, respectively (P<0.05; n=5). These results indicate that raising mucosal glucose increases glucose in ASL and that Staph. aureus utilises this glucose to promote their growth. Moreover, by reducing tight junction integrity bacteria may also increase the paracellular diffusion of glucose into the ASL.

PC195

The mucosal glucose concentration of polarised human airway epithelial cell monolayers affects luminal Staphylococcus aureus growth

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Glucose in the airway surface liquid (ASL) is normally maintained at a low concentration compared to glucose levels in the blood (~12.5 times lower). In healthy humans, ASL glucose concentrations rise in response to increased blood glucose (1). Patients on intensive care with elevated ASL glucose are more likely to have respiratory infection, particularly with methicillin-resistant Staphylococcus aureus (MRSA; 2). The aim of this study was to investigate the relationship between mucosal glucose concentration, glucose in the ASL and bacterial growth using an in vivo model of human airway epithelium. H441 epithelial cells were grown on transwell inserts for 10 days, at air-liquid interface, to form polarised monolayers. Cells were washed and bathed in a HCO3 buffered Krebs solution to remove antibiotics and growth supplements which could effect bacterial growth. The glucose concentration of the mucosal Krebs solution was set at 10, 20 or 40mM. For ASL glucose analysis, cells were incubated for 1 hour with the different glucose concentrations. 150μl of solution containing 0.67μg ml⁻¹ FITC-dextran was used to wash the surface of the transwells. Glucose concentration of the washes was quantified using a fluorometer. ASL volume was calculated by fluid displacement. For co-culture studies, Staph. aureus (8325-4) were grown overnight in RPMI medium to produce a culture of ~10⁶ colony forming units (CFU) ml⁻¹. 50μl of 10⁷ CFU ml⁻¹ in glucose-free RPMI was applied to the luminal surface of H441 monolayers. The H441-Staph. aureus co-culture was incubated for 7 hours at 37°C with 5% CO2. Transepithelial electrical resistance (TEER) of the monolayers was measured using a voltohmmeter (WPI). The co-culture was then scraped from the transwells and CFU's were determined using the Miles-Misra method. Calculated ASL glucose was less than mucosal glucose concentration but increased from 0.5±0.2mM to 1.5±0.2 and 6.5±1.3mM as basolateral glucose concentration was raised from 10mM to 20mM and 40mM, respectively (P<0.05; n=3-5). The TEER of H441 monolayers was significantly reduced following the addition of Staph. aureus (248±44 Ω cm²), compared to control monolayers (498±104 Ω cm²; P<0.05; n=5). Altering mucosal glucose had no effect on H441 TEER. Luminal Staph. aureus growth directly correlated with mucosal glucose concentration, increasing from 8.6±8.1x10⁷ to 1.5±0.8x10⁸ CFU ml⁻¹ and 3.4±1.4x10⁸ CFU ml⁻¹ upon elevation of mucosal glucose from 10mM to 20mM and 40mM, respectively (P<0.05; n=5). These results indicate that raising mucosal glucose increases glucose in ASL and that Staph. aureus utilises this glucose to promote their growth. Moreover, by reducing tight junction integrity bacteria may also increase the paracellular diffusion of glucose into the ASL.


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Poster Communications
The natural antioxidant cinnamtannin B-1 reduces the effects of H2O2 on CCK-8-evoked responses in mouse pancreatic acinar cells

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Intracellular Ca2+ overload and oxidant production play a key role in the intracellular activation of digestive enzymes leading to the development of pancreatitis. Cinnamtannin B-1 is a naturally occurring A-type proanthocyanidin, which belongs to a class of polyphenols that is widely distributed throughout the plant kingdom. This compound shows antioxidant properties. The aim of our study was to examine the antioxidant properties of cinnamtannin B-1 against hydrogen peroxide (H2O2) effects in mouse pancreatic acinar cells. Cells were prepared by collagenase digestion of the pancreas, following previously described methods (Gonzalez et al., 1997). We have analyzed cytosolic free Ca2+ concentration ([Ca2+]c), cell oxidative state, amylase secretion and viability of pancreatic acinar cells treated with cinnamtannin B-1 in the presence of various concentrations of H2O2. Cells were loaded with the fluorescent ratiometric Ca2+ indicator fura-2 to monitor [Ca2+]c by single cell analysis of fluorescence. Cell oxidative state was measured in a spectrofluorometer, following changes of fluorescence of CM-H2DCFDA-loaded mouse pancreatic acinar cells. Amylase release was determined using the Phadebas blue starch colorimetric method, employing a spectrophotometer. Cellular viability was determined following reduction of alamarBlue® and employing an ELISA spectrofluorimeter. Our results show that H2O2 (0.1 – 100 μM) lead to an increase in fluorescence of CM-H2DCFDA, reflecting an increase in oxidation. Cinnamtannin B-1 (10 μM) reduced H2O2-induced oxidation of CM-H2DCFDA. CCK-8 induced oxidation of CM-H2DCFDA similarly to the low micromolar concentrations of H2O2, and cinnamtannin B-1 reduced the oxidant effect of CCK-8. In addition, H2O2 induced a slow progressive increase in [Ca2+]c. Cinnamtannin B-1 reduced the effect of H2O2 on [Ca2+]c but only at the lower concentrations of the oxidant. CCK-8 induced a biphasic effect on amylase secretion, resulting in a maximum at 0.1 mM, and a reduction of secretion at higher concentrations. H2O2 inhibited amylase secretion in response to CCK-8. Preincubation of cells with cinnamtannin B-1 reduced the inhibitory action of H2O2 on CCK-8-evoked enzyme secretion. Finally, H2O2 reduced cell viability and the antioxidant protected acinar cells against H2O2. In conclusion, the beneficial effects of cinnamtannin B-1 appear to be mediated by reducing the intracellular Ca2+ overload, ROS production and intracellular accumulation of digestive enzymes evoked by ROS, which is a common pathological precursor that mediates acute pancreatitis. Our results support the use of natural antioxidants in the therapy against oxidative stress-derived deleterious effects on cellular physiology.


This work was supported by Junta de Extremadura-FEDER (P080A018 and GR10010).

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Intestinal fluid movement is driven by osmotic gradients that are, in turn, established by active ion transport across the epithelium. Fluid absorption normally predominates and is promoted by Na+ absorption, while fluid secretion is driven by Cl- secretion. Epithelial transport function is dependent on cellular O2 and energy supply and our previous studies have shown that prolyl hydroxylases (PHDs), which function as cellular O2 sensors, are important regulators of intestinal fluid and electrolyte secretion in vitro and in vivo. However, little is known of the role of PHDs in regulating intestinal absorptive function. Here, we sought to investigate the role of PHDs in regulating epithelial sodium channel (ENaC) activity and expression. The pan-hydroxylase inhibitor dimethylloxylglycine (DMOG) was used to inhibit hydroxylases. ENaC activity was measured as amiloride-sensitive changes in short circuit current (Isc) across monolayers of T84 cells pretreated with 4-phenyl butyrate (4-PBA; 5 mM) to induce ENaC activity, or across muscle-stripped segments of rat rectum. Sodium glucose transporter-1 (SGLT1) activity was measured as phloridzin-sensitive changes in ISc across segments of mouse jejunum. Western blotting was used to assess protein expression. Treatment of T84 cell monolayers with 4-PBA (5 mM; 24 hrs) led to an induction of ENaC expression and an increase in basal ISc of 17.8 μA/cm2 that was wholly sensitive to the ENaC blocker, amiloride (10μM). Pretreatment of the cells with DMOG (1mM, 24 hrs) significantly reduced the 4-PBA-induced current by 29 ± 6.3 % (n=5, p < 0.01 by paired t-test). This reduction in activity was not due to altered abundance of the ENaCα subunit as shown by western blot analysis (n=3). Furthermore, treatment of rats by intraperitoneal injection of DMOG (40mg/kg) significantly reduced the amiloride-sensitive current across ex vivo rectal tissue to 64 ± 13.3% of that in control tissues when measured 24 hrs after treatment (n=6; p = 0.05 by paired t-test). The effects of hydroxylase inhibition were specific for Na+ absorption through ENaC since treatment of mice with DMOG failed to alter glucose (25 mM)-stimulated SGLT1 activity in ex vivo sections of jejunum when measured 24 hrs after treatment (n=6). Conclusions: These studies demonstrate a novel role for PHDs in regulating intestinal absorptive function. PHD inhibitors specifically downregulate large intestinal ENaC activity while small intestinal SGLT-1 activity is unaltered. Mechanisms underlying DMOG inhibition of ENaC function remain to be elucidated but do not appear to involve alterations in expression of the ENaCα subunit of the protein. Our data suggest that PHDs may provide a new target for development of drugs to treat intestinal disorders associated with dysregulated epithelial absorptive function.

Supported by Science Foundation Ireland RFP Award to S.J. Keely.
The nuclear bile acid receptor, farnesoid X receptor, inhibits CFTR expression and Cl− secretion in colonic epithelial cells

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Cl− secretion, the primary driving force for fluid secretion in the intestine, can become dysregulated in many pathological conditions, leading to onset of diarrhoea. However, drugs which directly target epithelial transport proteins for treatment of diarrhoeal diseases are still lacking. We have previously shown that activation of the nuclear bile acid receptor, farnesoid X receptor (FXR), potently inhibits epithelial Cl− secretion in vitro and in vivo, an effect which is mediated by a decrease in Na+−K+−ATPase activity, without altering cellular expression of the protein. Here, we sought to investigate the effects of FXR activation on the activity and expression of CFTR, the primary exit pathway for Cl− in epithelial cells. Methods: GW4064 was used as a prototypical FXR agonist. T84 cell monolayers were mounted in Ussing chambers and Cl− secretion was measured as changes in short-circuit current. Protein expression was measured by RT-PCR and western blotting. Data are presented as mean ± SEM and were statistically analysed by ANOVA or Students t-tests as appropriate. Results: As previously reported, GW4064 (5 μM) inhibited Cl− secretory responses to the Ca2+- and cAMP-dependent agonists, carbachol (CCh) and forskolin (FSK) to 63 ± 8 (n = 8; p < 0.01) and 60 ± 7% (n = 8; p < 0.001) of control values, respectively. Under experimental conditions that specifically isolate Cl− currents across the apical membrane, FSK-induced responses were attenuated 90 ± 2% by the CFTR inhibitor, CFTR-inh-172 (10 μM) (n = 4), confirming these cAMP-dependent currents to be mediated by CFTR. Interestingly, GW4064 (5 μM) also decreased CFTR-mediated currents to 76 ± 5% (n = 8; p < 0.01) of those in control cells, measured 5 min after FSK stimulation. Furthermore, western blot and RT-PCR analysis revealed that GW4064 (5 μM) inhibited CFTR protein expression to 33 ± 9% (n = 4; p < 0.01) of that in control cells without altering CFTR mRNA levels (n = 3). Finally, GW4064 did not affect basal or FSK-stimulated cAMP levels in T84 cell monolayers which were 78 ± 21 and 96 ± 2% of those in control cells, respectively (n = 4). Conclusions: These data reveal a novel role for FXR in regulating the expression and activity of epithelial CFTR Cl− channels and have important implications for our understanding of the role of bile acids in regulating intestinal fluid and electrolyte transport. Our data suggest that by virtue of its ability to inhibit multiple components of the Cl− secretory pathway, FXR represents a good target for the development of new anti-diarrhoeal drugs.

Funded by a Principal Investigator Award from Science Foundation Ireland to SJK.

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The effect of an exercise ball on trunk muscle responses to rapid limb movement

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Anticipatory postural control is a key component of human movement and has been shown to be significantly impaired following disorders of neurological (Dickstein et al., 2004) and musculoskeletal systems (Hodges & Richardson, 1996). The use of exercise balls is widely advocated as an aid to facilitate improvements in postural control in patients with trunk muscle weakness, but its effect on anticipatory postural control has received little attention.

This study compared the latency and amplitude of trunk electromyographic (EMG) activity in response to limb movement when seated on an exercise ball or on a chair in sixteen healthy, moderately active human subjects. At the sound of an auditory cue, the subjects carried out either hip flexion or arm flexion (unilateral or bilateral), with or without weights, as fast as possible, while sitting on an exercise ball or a standard chair. The latency and amplitude of EMG activity were recorded from selected trunk muscles: erector spinae (ES), external obliques (EO), internal obliques (IO), rectus abdominis (RA) and either an upper limb muscle (deltoid) or a lower limb muscle (rectus femoris).

There were minimal differences in amplitudes of EMG activity in any of the trunk muscles between the conditions (ball or chair) following the upper limb movements. These results suggest that there is no benefit in simple arm flexion movements whilst seated on the exercise ball in comparison to a chair. The amplitude of the RA (p< 0.05), EO (p<0.01) ipsilaterally and IO (p <0.05) bilaterally was significantly higher on the ball in the weighted hip flexion protocol. In addition, there is some suggestion of early activation of RA and EO muscles in the weighted hip flexion condition.

These results indicate that hip flexion activity while seated on a gym ball elicits earlier anticipatory postural activities while upper limb flexion does not. This result may have implications for rehabilitation of postural and movement deficits in people with conditions such as stroke.


The authors acknowledge the support of both King’s College and Imperial College London for the funding for this study.

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An exercise test to characterise the domains of aerobic function in humans

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The consistent physiological signal for impending exercise intolerance is the failure of oxygen uptake (VO₂) to meet the energy demands of the task in a steady state [1]. The upper limit for steady state exercise is demarked by critical power (CP), which is the asymptote of the hyperbolic relationship between power output and tolerable duration [2]. Together with the lactate threshold (LT) and VO₂max, CP partitions the domains of aerobic function that cluster power output ranges by their common physiological response characteristics [3]. There is currently no exercise testing protocol, however, to determine LT, CP and VO₂max in a single laboratory visit. Given that CP is the highest sustainable power output following intolerance at VO₂max [4], we reasoned that a maximum sustained effort immediately following standard ramp-incremental exercise [1] would allow the domains of aerobic function to be estimated in a single test. Eight healthy men (21-30 yr) performed a series of cycle ergometry (Excalibur Sport, Lode, NL) protocols. Following familiarisation participants performed ramp exercise to intolerance with the incrementation rate computer-controlled at 20 W/min, immediately followed by a 3-min all-out ‘sprint’ (ramp-sprint test; RS) for the measurement of cadence-dependent power output (CP₉₀%). On separate days participants performed a series of 4-5 constant-power tests (gold-standard method; GS [2]) to estimate CP₉₀ and VO₂₂₅max-GS. Subjects also completed constant-power tests (for 30-min or intolerance) at work rates corresponding to 10 W below LT and CPRS, and 10 W above CP₉₀ to verify the steady state exercise. Breath-by-breath VO₂ and capillary blood [lactate] were measured using a mass-spectrometer and turbine (MSX, NSpire, UK) and enzymatic oxygen-rate method (GM7, Analox, UK). From these measurements CP and VO₂max were compared (t-test) between RS and GS tests. LT averaged 2.0±0.3 L/min (mean±SD). CP₉₀ (255±52 W) and VO₂₂₅max (4.0±0.7 L/min) did not differ (p = 0.8 and 0.7) from CP₉₀ (256±48 W) and VO₂₂₅max (4.0±0.6 L/min). Exercise 10 W below LT and CPRS was sustained for 30±0 min with VO₂ and blood [lactate] in a steady state. Exercise 10 W above CPRS caused the attainment of VO₂max (4.0±0.6 L/min) and peak [lactate] (11±2 mM) in 20±5 min. Consistent with the hypothesis the RS test accurately identified all three indices of aerobic function with a precision of 8, 3 and 4% (for LT, CP and VO₂max), which compares favourably to current methods [1,2,4]. The single-visit RS test also provided an estimation of the upper steady state limit to within 4%. These data suggest that the domains of aerobic function may be reliably estimated from a single maximal exercise test with non-invasive measurements. The RS test may, therefore, simplify the characterisation of aerobic function and monitoring of exercise training, therapeutic and/or experimental interventions.

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Diurnal variation in the circulatory changes associated with initial orthostatic hypotension during active standing: sleep vs. circadian influences

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During everyday activities, the incidence of vasovagal-related syncope is substantially higher in the morning compared to other times of day. In 3-10% of the general population, standing is one such activity which has been identified as a trigger of syncope. In physiological terms, a critical time is during initial orthostatic hypotension (IOH), involving a transient fall in systolic and/or diastolic blood pressure of >40 and 20 mm Hg respectively, within the first 15 s of standing (Wieling et al., 2007). We have reported that there is a lack of diurnal variation in the physiological responses associated with this IOH (Lewis et al., 2011). However, subjects in this study slept normally before the morning trial but not before the afternoon trial. Therefore, the aim of this study is to examine the diurnal variation in the cardiorespiratory and cerebrovascular physiological responses during IOH using a protocol in which the amount of prior sleep is controlled. Nine healthy individuals, aged 27±3 yrs, completed the protocol at 06:00 h and 16:00 h in a randomized order. At each time of day continuous beat-to-beat recordings of mean MCAv, MAP, SV, cardiac output (Q), heart rate (HR), and end-tidal carbon dioxide (PETCO2) were obtained during supine rest and active standing (3 min).

Importantly, approximately 4 h of sleep preceded both the morning and afternoon test. Differences between times of day were analysed using paired t-tests and reported as mean±SE. All variables measured at baseline when resting supine did not differ between times of day (P>0.10), except HR which was 4±2 beats·min−1 higher in the afternoon (P=0.02). All individuals (n=8) experienced IOH during active standing at both times of day. The initial decline in MAP occurred within 9±1 s at both times of day (P=1.0); however, the magnitude of decline in the afternoon (47±3 mm Hg) was greater than the morning (34±3 mm Hg; P=0.01). The magnitude of decline in mean MCAv was similar in the morning (24±8 cm·s−1) and afternoon (27±9 cm·s−1; P=0.51). No diurnal variation was evident in MAP measured 30 s after standing, although Q was 1.2±0.4 L·min−1 greater in the afternoon at this time (P=0.03). These data indicate that the magnitude but not the time-course of the transient decline in MAP during IOH alters with time of day. Because we controlled the amount of sleep prior to each time of day, the greater decline in MAP during active standing in the afternoon may be under the control of endogenous circadian rhythms. Nevertheless, despite the greater hypotension in the afternoon, cerebral perfusion was well controlled at both times of day. The finding is suggestive of possible compensatory interactions between blood pressure and mechanisms of cerebral blood flow control.

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muscles have not been examined here, but might include motorneurone cell death, decreased myosin content, post-translational modifications of the myosin molecules and/or increased intramuscular adipose and connective tissue. funded by EC FP7: MYOAGE (223576)

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Effect of exercise induced Stress on leukocyte Hsp72, GRP78, Hsp90α and GRP94 mRNA expression

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Heat shock proteins (HSPs) are a group of proteins which are ubiquitously expressed within leukocytes and which have a key function in maintaining leukocyte homeostasis and function during both the immune and inflammatory responses to exercise. They function to prevent a loss of protein activity caused by various physiological stressors such as exercise, heat and oxidative stress (Noble et al, 2008). Exercise induced upregulation of basal Hsp content may provide enhanced protection against future stressors (Paulsen et al, 2009), however there is little information regarding the effect of mechanical stress (exercise induced muscle damage) and heat stress on expression across the whole range of Hsps. The aim of this study was therefore to determine whether metabolic stress, mechanical stress, heat stress or heat plus mechanical stress altered leukocyte hsp gene expression. With university ethical approval 14 physically active students (mean ± SD; age 19.86±2.53 y, body mass 71.33±8.66 kg, VO2max 55.49±5.94 ml.kg-1.min-1) participated. Using an independent groups design, participants were randomly allocated to either a temperate (20°C, 50% RH) or heat group (30°C, 50% RH). In a random order participants in the temperate group completed 30 min running (1% gradient) at lactate threshold (LT; metabolic stress/control condition) and 30 min downhill running (-10% gradient) at LT (mechanical stress). Participants in the heat group completed the same protocols in the heat (heat stress and heat plus mechanical stress). Core temperature, oxygen consumption, heart rate and subjective RPE & ratings of thermal sensation (TS) were measured continuously during exercise. Venous whole blood samples were obtained for determination of Hsp gene expression along with measurements of muscle soreness (pressure sensitivity and subjective ratings) immediately before, immediately after, 3hrs after and 24 hrs after exercise. Leukocytes were isolated using an erythrocyte lysis solution, RNA was extracted via the Trizol method and gene expression was determined using Quantitative RT-PCR. Initial statistical analysis was conducted using a factorial ANOVA. Initial results below equate to N=5. Heat & mechanical stress induced a significant (380.4±112.89% Vs 152±43.84% P <0.05) increase in hsp72 gene expression and a non significant increase in GRP78 (224.6±106.7% Vs 121.43±18.03%) hsp90α (359.6±230.72% Vs 121.83±16.13%) and GRP94 gene expression (393.4±214% Vs 118.2±75.5%) compared to metabolic stress. Core Temperature was significantly elevated in Heat & mechanical stress (39.2±0.2°C) compared to metabolic stress (38.3±0.3°C) suggesting that an elevated core temperature may have contributed to the increase in hsp gene expression.


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Muscle to Bone ratio as an intra-individual indicator of sarcopenia

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The decline in muscle mass and strength during ageing and its consequences for health and quality of life have been well documented. However, the data on which this is based are largely from cross-sectional studies and it is difficult to acquire longitudinal data over a 70-year time span. The implicit assumption of the cross-sectional studies is that the muscles of the older subjects in their twenties were of a similar size to those of the present-day 20-year olds. The challenge is to find a way of relating the present state of the muscle to what it might have been in the past by relating the muscle size to some standard that shows little, if any, changes in adulthood. The femoral cross sectional area might be such a standard as it changes little, across the adult lifespan. Therefore, the muscle mass to bone area ratio might be an indicator of extent of age-related muscle atrophy.

To investigate this, 21 young (22.4±3.0 yrs) and 20 elderly (71.4±4.6 yrs) men completed an MRI scan (Esaote, 0.25-T; G-Scan) and maximal isometric voluntary knee extension contractions (MVC). For MRI, cross-sectional slices were obtained perpendicular to the thigh at 25mm intervals from the distal the proximal ends of the femur using a turbo-3D T1-weighted protocol. Quadriceps muscle (Qcsa) and femur cross sectional area (Fcsa) were determined in each slice. The maximum Qcsa may be representative for the maximal force the muscle can generate, while the risk of fracture might be highest where the Fcsa is smallest. Therefore the ratio between the smallest Qcsa and maximum Qcsa was represented as the muscle: bone ratio. Student’s t-tests were used to compare the groups and Pearson correlation coefficients were used to determine relationships between variables. Data are presented as mean ± SD. We observed no difference in smallest Qcsa (Y: 6.09±0.8cm2 vs. O: 6.27±0.87 cm2 p=0.52), cortical (p=0.86) or trabecular (p=0.46) bone CSA between young and old. Compared with young, elderly had a smaller maximal Qcsa (p<0.0001) and MVC torque (O: 160±34 Nm vs. Y: 263±44 Nm, p<0.0001). The muscle: bone ratio in elderly was 9.6±1.4 and in young it was 14.0±1.5 conferring a 32% lower muscle: bone ratio in elderly men (p<0.0001). Muscle: bone ratio correlated significantly in both Y and O groups (see figure; p<0.01).

In conclusion, we report here that muscle and bone cross sectional areas are correlated in young subjects. The fact that femur cross sectional area was similar in young and older subjects while muscle size was smaller in old suggests that the bone size may be used to determine the extent to which the
older muscle has deteriorated with age. This circumvents, to some extent, the difficulty of conducting long term longitudinal studies of muscle deterioration with age. The same technique may also be useful in clinical studies of conditions where muscle atrophy can occur.

Funded by EC FP7 Myoage(223576)

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PC206

Neurocognitive function across the spectrum of high contact sports

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Neurocognitive function (NF) is thought to be impaired in individuals who suffer repeated blows to the head. Similarly, rugby players who have lost consciousness ≥ 3 times have a significantly reduced neurological performance to those who have no history (Gardner et al, 2010). Neurocognitive tests examine memory, mental agility and co-ordination, and are a sensitive method for detecting acute traumatic brain injury (TBI) (Capruso et al, 1992). In the current study, we hypothesised that neurocognitive function would be impaired in boxers and rugby players with a history of loss of consciousness (LOC), with the greatest impairment in boxers, relative to healthy controls.

Eight currently active professional male boxers aged 29 (mean) ± 3 (SD) years and 9 male rugby players (23±3 years) all with a history of LOC (4±4 LOC in 137±97 competitive rounds and 4±5 LOC in 13±6 playing years, respectively) were compared to seven physically active non-concussed male controls (30±7 years). A battery of psychometric tests was employed to measure NF, separated into 3 subcategorises; Memory: Ray Auditory Verbal Learning Test (RAVLT) split into 3 sections, total number of words remembered (A1-A5), total recalled from memory from initial list (A6) and Digit Span Test forwards and backwards (RDF & RDB). Mental Agility: Trail Making Tests, A (TMTA) and B (TMTB) and the Digit Symbol Substitution Test (DSST). Visual-motor Coordination: Groove Pegboard Dexterity Test, using dominant (GPD) and non-dominant (GPND) hands. After confirmation of normality using Shapiro-Wilk W tests, data were analysed using a one-way ANOVA and Bonferroni corrected independent samples t-tests. Significance was set at P<0.05. NF was clearly more impaired in boxers vs controls, whilst only partially declining in rugby players (Table 1).

These findings demonstrate that NF is progressively more impaired across the spectrum of contact sports and justifies its utility to diagnose.

Table 1. Neurocognitive function

<table>
<thead>
<tr>
<th>Neurocognitive Function Tests</th>
<th>Boxing (n=8)</th>
<th>Rugby (n=9)</th>
<th>Controls (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAVLT (A1-A5) (No.)</td>
<td>338±113</td>
<td>44±128</td>
<td>96±44</td>
</tr>
<tr>
<td>RAVLT (A1-A5) (Yes.)</td>
<td>335±114</td>
<td>44±104</td>
<td>97±44</td>
</tr>
<tr>
<td>RAVLT (B) (No.)</td>
<td>371±121</td>
<td>65±27</td>
<td>82±27</td>
</tr>
<tr>
<td>RAVLT (B) (Yes.)</td>
<td>371±121</td>
<td>65±27</td>
<td>82±27</td>
</tr>
<tr>
<td>TMTA (Sec)</td>
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<td>57±10</td>
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</tr>
<tr>
<td>GPND (Sec)</td>
<td>60±14</td>
<td>65±3</td>
<td>53±14</td>
</tr>
</tbody>
</table>

Mean ± SD

*P<0.05 Boxers Vs Controls, †P<0.05 Boxers Vs Rugby, ‡P<0.05 Rugby Vs Controls


The present research was supported by the JPR Williams Trust, in co-ordination with the University of Glamorgan.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC207

Inverse relationship between blood-borne and expired nitric oxide during exercise: an interpretative dilemma

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Vascular nitric oxide (NO) is a critical molecule involved in the regulation of vascular function. However, its measurement is complex due to its short half-life and high reactivity. Given that blood is in contact with the vascular endothelium, blood-borne NO should be considered the “gold standard” measurement for its quantification. The reliance on the more distal measurement of exhaled NO may therefore be misrepresentative of NO bioavailability. To examine if any association exists between these two sites of measurement (blood vs. exhaled) the current study aimed to simultaneously measure exhaled and venous nitrate (NO2−) in response to an acute exercise challenge.

Exhaled NO and venous NO2− was quantified before and after a maximal exercise challenge in seven apparently healthy males 35 (mean) ± 7 (SD) yrs. Venous blood was collected from an indwelling catheter placed in an antecubital vein and was immediately centrifuged (2500rpm; 4°C). Plasma was flash frozen and stored at -80°C before later analysis. Both exhaled NO and plasma NO2− concentrations were measured using from a new list (B1) and total recalled from memory from initial list (A6) and Digit Span Test forwards and backwards (RDF & RDB). Mental Agility: Trail Making Tests, A (TMTA) and B (TMTB) and the Digit Symbol Substitution Test (DSST). Visual-motor Coordination: Groove Pegboard Dexterity Test, using dominant (GPD) and non-dominant (GPND) hands. After confirmation of normality using Shapiro-Wilk W tests, data were analysed using a one-way ANOVA and Bonferroni corrected independent samples t-tests. Significance was set at P<0.05. NF was clearly more impaired in boxers vs controls, whilst only partially declining in rugby players (Table 1).
established ozone-chemiluminescence techniques (Sievers NOA 280i).

Acute exercise elicited no change in either exhaled NO (Pre: 19 ± 7 vs. Post: 19 ± 6 ppm; P > 0.05) or plasma NO2. (Pre: 309 ± 45 vs. Post: 376 ± 121 nmol; P > 0.05). No statistical relationship was observed between delta changes (exercise minus rest) in exhaled NO and plasma NO2; however, there was a week inverse relationship between the two (r = -0.732, P = 0.06) (Figure 1).

NO biochemistry is a very complicated field and many physiological parameters affect its availability and physiological function. Exhaled NO has become a popular way of assessing its bioavailability, however, the use of this technique must warrant caution. Current data tentatively suggests that exhaled NO may not reflect vascular NO bioavailability and therefore be an un-reliable marker of no activity.

Figure 1. Relationship between changes in exhaled NO and venous plasma nitrite.

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Similar cardiovascular and metabolic adaptations to exercise training in older men and women with type 2 diabetes

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Improvements in peak oxygen uptake (VO2peak) following exercise training in healthy older individuals are not sex-specific. However, studies suggest greater levels of cardiovascular adaptations occur in males, with a greater level of O2 extraction in females ¹. This human study examined if cardiovascular and metabolic adaptations to exercise training were sex-specific in individuals with type 2 diabetes (T2D).

25 male (M) (EXS n=13; CTL n=12) & 19 female (F) (EXS=10; CTL=9) age- and BMI-matched individuals with T2D completed testing. The exercise groups were aerobically & resistance trained three times per week for 12 weeks. Before and after the intervention, subjects underwent a graded incremental cycle test to assess ventilatory threshold (VT) and VO2peak. VO2 kinetic responses to steady-state cycling at 80% of their current ventilatory threshold (VT) were determined by fitting a bi-exponential function to the VO2 data. Cardiac Output (inert gas rebreathing) (CO) was assessed at rest, 30 seconds and 4 minutes. Data were analysed using a 3-way repeated measures ANOVA, with significant differences detected using the Bonferroni post hoc test.

Exercise resulted in a significant improvement (P<0.05) in VO2peak (EXS M: (mean ± sd) 26.8 ± 4.2 vs. 29.5 ± 4.1; EXS F: 19.8 ± 5.3 vs. 22.9 ± 4.7 ml.kg⁻¹.min⁻¹). The magnitude of increase in VO2max (EXS M: 10.61%; EXS F: 16.91%) was similar between groups. Training resulted in a trend towards faster time constant values for the 2nd phase of the VO2 kinetic response (P = 0.095). Males demonstrated higher CO and stroke volumes (SV) than females at all time points, but exercising males and females did not show any significant improvements in cardiovascular parameters following the intervention compared to baseline (table 1). The control groups did not demonstrate any significant differences.

Consistent with healthy individuals, peak performance improved by a similar magnitude in males and females with T2D following the training intervention. However, the lack of gender specific differences in VO2 kinetic, CO and SV responses during steady-state exercise suggest that the effect of training in performance in males and females following training was driven by a similar level of cardiovascular adaptations, differing from findings in non-diabetic individuals.

Poster Communications

Oxygenation During Acute Hypoxia

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A central debate in exercise science literature is the nature of fatigue (central and/or peripheral) that determines exercise performance under different conditions (3). Hypoxia has a small but direct role on the cessation of exercise, specifically during endurance performances (1, 2). Furthermore, the pacing strategy is thought to be altered when the endpoint is falsified and the external environment is known to alter physiological variables (4). In this study we examined the effects of hypoxia and pacing strategy on central (brain) and peripheral (muscle) changes in trained cyclists. Cyclists (n=10; ages = 27.3 ± 6.25yrs) performed a maximal aerobic capacity (VO2max= 52.99 ± 6.88ml/kg/min) test prior to two 20km time trials (20TT) in randomly assigned order; one in normoxia (N; 21% O2) and one in acute hypoxia (H; 15% O2). Participants were blinded to time, distance, grade of incline, and condition. Near infrared spectroscopy (NIRS) was used continuously to moni-
Resistance exercise training improves microvascular blood volume in response to feeding

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Insulin and nutrient delivery to the muscle microvasculature are rate limiting for insulin action, and possibly anabolic responses to nutrients. Both muscle contraction and feeding (insulin) can increase total blood flow and microvascular blood volume (MBV) in skeletal muscle (1). Since exercise is associated with improvements in metabolic function (i.e., insulin sensitivity) we hypothesised that resistance exercise training (RET) would sensitize the muscle microvasculature to feeding. In preliminary studies we recruited 6 healthy male subjects (58.8±9.8 y, BMI: 26.9±1.0 kg.m²) 3 of whom underwent 20 weeks of supervised whole body RET with the remainder acting as untrained (UT) controls. Leg blood flow (LBF) was measured using Doppler Ultrasound and MBV by contrast enhanced ultrasound (CEUS). Measures were made: (i) in the postabsorpive, rested state, where subjects had fasted for 12 h and remained in a supine position for 50 min, and (ii) in the fed state (Glamin: prime; 34 mg.kg⁻¹, continuous dose; 102 mg.kg.hr⁻¹ and 20% dextrose infused at a rate to maintain blood glucose between 7.7-7.5 mmol.l⁻¹). Under both postabsorptive and fed conditions, LBF was measured over 40 min before an infusion of Definity™ perflutren microbubbles (1.5 ml in 18.5 ml 0.9% saline, 1.2 ml.min⁻¹ (2)). After 9 min to allow attainment of steady state bubble concentration, microbubbles were destroyed by high energy ultrasound and refilling of the microvascular space (representing MBV) was imaged during 4 consecutive 45 s recordings and data was analysed using Q-lab software. LBF is reported in L.min⁻¹ whereas MBV is percentage change in acoustic intensity (AI) area under the curve (AUC); data was analysed using an unpaired t-test and reported as means±SE. There was no effect of RET on postabsorptive LBF (UT: 0.48±0.04, RET: 0.38±0.01 L.min⁻¹) or on fed state LBF (UT: 0.55±0.09, RET: 0.41±0.07 L.min⁻¹) which did not increase after feeding in either group (UT: +11.58±8.52%, RET: +7.56±16.63%). In contrast, MBV was significantly greater in response to feeding only in the RET group (UT: +7.8±11.4%, RET: +68.9±17.7%, P<0.05). We conclude that RET improves MBV responses to feeding even in the absence of significant changes in LBF. Improvements in MBV after RET may underlie beneficial metabolic adaptation to exercise, such as improved insulin sensitivity and anabolic responses to nutrients.

PC210

Microvascular reperfusion images from (A) postabsorptive and (B) fed conditions.


This work was supported by a grant from the Dunhill Medical Trust.

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Sarcopenic muscle shows distinct sex differences at the transcriptional level


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Introduction: Age-related loss of muscle mass (sarcopenia) is associated with several adverse outcomes including increased frequency of falls (Szulc et al 2005). An operational definition of sarcopenia is important in order to obtain comparable measures of prevalence, determine relative risk, target patients for therapeutic intervention, set treatment goals and monitor outcome. Current definitions of sarcopenia are based upon muscle mass referenced to young adult reference data (Cruz-Jentoft et al 2010). However no studies have attempted to validate this definition using muscle molecular biology. We hypothesised that (i) skeletal muscle gene expression would be different in the presence of sarcopenia and (ii) sex differences in the muscle transcriptomic signature would exist between elderly sarcopenic and non sarcopenic individuals.

Methods: We studied 16 healthy men (n=8; mean 77 y (SD ± 0.8)) and women (n=8; 79 y (SD ± 1.2)) who were classified as sarcopenic/ non-sarcopenic on the basis of quadriiceps MRI cross-sectional area (CSA) 2SD below young adult mean values for men and women (21.3 cm².m⁻² and 17.3 cm².m⁻² respectively). We obtained samples of quadriiceps muscle obtained by needle biopsy (under local anaesthetic using 1% lidocaine). Global expression profiles were examined using Affymetrix hgu133plus2 GeneChip arrays. After non-specific filtering, the intensity of each probe was correlated with quadriiceps CSA (cm².m⁻²) and probes demonstrating Pearson rho > 0.5 and p-value < 0.05 were selected for further analysis (325 genes in men and 418 in women). The array data for the correlating genes were used to drive clustering of the samples by euclidean distance. We examined correlating genes in both men and women for gene ontology biological processes (GOBP) and membership of pathways (KEGG).

Results: Hierarchical clustering of the array data for correlating genes revealed 2 broad clusters for both men and women around the MRI derived sarcopenia cut-off values. Informatic analysis of the correlating genes revealed marked sex differences. In men there was a strong representation for processes involved in axonogenesis and oxidative stress responses. Interestingly, the gene ontology term ‘ubiquitin mediated proteolysis’ correlated negatively with muscle CSA. In women, indicators of re-modelling (muscle tissue morphogenesis) and muscle contraction processes were strongly represented. The focal adhesion and Jak-STAT pathways were enriched.

Conclusion: We have used transcriptomics to validate the operational definition of sarcopenia. Our data suggest the mechanisms underlying human sarcopenia may be different in men and women.


Neurovascular coupling and distribution of cerebral blood flow during exercise

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There is a ~20% increase in global cerebral blood flow (CBF) during exercise that is thought to be principally due to increases in neural activation. There is evidence, however, that the increased CBF may be different between the anterior and posterior cerebral territories, perhaps because neuronal activation is locally coupled to changes in blood flow. This neurometabolic coupling is termed neurovascular coupling (NVC), and can be assessed with simple visual stimulation tasks, such as reading. It is not understood, however, how changes in baseline CBF influence this regional blood supply regulation, and also if exercise-induced CBF increase is a global or regional phenomenon. In ten healthy subjects (5 male; aged 25 ± 6 years), cerebral blood velocity was measured in the middle cerebral artery (MCA) using transcranial Doppler ultrasound in the posterior and middle cerebral arteries (PCAv and MCAv, respectively) were recorded. The P1 segment of the MCA was located on the right and left hemispheres, and the first signal used for measurement of MCA blood velocity. The MCAv was measured on the contralateral side 10mm distal to the MCA/anterior cerebral artery bifurcation. Arteries were confirmed using ipsilateral carotid compression to verify a velocity reduction in the MCA and increased velocity in the PCA. Heart rate (ECG), beat-to-beat blood pressure (Finipres), and end-tidal PCO2 were recorded throughout. NVC was quantified as the change in systolic PCAv to a visual stimulus. Following 2 minutes of baseline measures, five cycles of 40 seconds reading, 20 seconds eyes-closed with concomitant measurement of PCAv and MCAv were completed during both rest and steady-state exercise on an upright stationary bike at ~60% of estimated maximal oxygen consumption. Although PCAv was shifted to higher velocities during exercise, the relative change in PCAv with visual stimulation, and time to peak response, was not different between exercise and rest (resting delta PCAv: 9.3 ± 4.4 cm/s, 11.5 ± 3.2 s to peak; exercise delta PCAv: 9.2 ± 3.8 cm/s, 9.1 ± 2.4 s to peak). At rest mean MCAv was 55.8 ± 10.3 cm/s and PCAv 38.0 ± 7.3 cm/s. With steady-state exercise, MCAv increased by 15.2 ± 13.6% from baseline versus 26.1 ± 22.5% for PCAv (P<0.05). These data indicate that there is a regionally heterogenous increase in CBF during exercise that does not impose any restriction to a visual-stimulus induced increase in occipital cortex blood flow; exercise does not disrupt neurovascular coupling. Since NVC has been found to be a useful marker of cerebrovascular disease, our findings provide the first documentation of NVC during exercise, and consequently provides a useful point of reference for comparison against pathology.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Changes in erythropoietin concentration induced by acute hypoxia do not predict acute hypoxic ventilatory response or performance changes following living high – training low

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Background and hypothesis: Upon exposure to hypoxia a series of adaptive responses are triggered to try to compensate the decrease in inspired PO2. For instance, minute ventilation increases within minutes of exposure (Powell et al., 1998) and increased circulating erythropoietin (Epo) concentration has been detected after durations as short as ~48min (Eckardt et al., 1989). Recent research has highlighted a possible link between these two parameters (Brugniaux et al., 2006). It is, however, noticeable that certain athletes respond better than others (Chapman et al., 1998). Because of its involvement in both ventilatory acclimatization and erythropoiesis (and in turn aerobic performance), we hypothesised that the Epo response to an acute hypoxic exposure could predict the outcome of both of these adaptive responses.

Methods: Thirty nine athletes (32 men, 7 women) out of 41 completed the entire study. These 39 athletes were exposed to a simulated altitude of 3,000m for 3hr prior to a LHTL camp. On a separate day we also tested their acute hypoxic ventilatory response (HVR). Maximal aerobic performance was measured prior to and immediately after the LHTL camp.
**Results:** Epo increased by 29.4 ± 28.2% [-20.5% - +105.7%] (P<0.001) following the acute exposure. There was no gender-difference in the increased Epo concentration (P=0.94). However, no correlation between Epo and either HVR or the changes in performance was observed.

**Conclusion:** We conclude that despite the tight relation between oxygen sensing and Epo secretion, the time-domain difference of the respective response precludes any clear relation between these parameters. Moreover, despite its clear role in the increase in performance following LHTL, Epo could not be predicted to yield the outcome of LHTL and ultimately tease out potential “good responders” from “bad responders”.


This study was supported by grants from the International Olympic Committee and the French Ministry of Sports.

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**PC215**

**Cerebral oxygen vasoreactivity after lipopolysaccharide infusion in healthy humans**


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Sepsis, the systemic inflammatory response to infection, is frequently complicated by brain dysfunction, which may involve disturbances in cerebral oxygen transport. We have previously established lipopolysaccharide (LPS) infusion as a human-experimental model of systemic inflammation that mimics the early stages of sepsis (Taudorf et al. 2007). In the present study, we hypothesised that LPS infusion impairs cerebral oxygen vasoreactivity (COVR).

Ten healthy male volunteers aged 23 (mean, SD 2) years were enrolled in the study. A catheter was inserted in the radial artery, and volunteers underwent a four-hour intravenous infusion of Escherichia coli LPS (total dose of 2 ng/kg). Prior to the infusion and immediately after, cortical oxygenation and middle cerebral artery blood flow velocity (MCAv) were measured by dual-wavelength near-infrared light spectroscopy and transcranial Doppler ultrasonography, respectively. During measurements, three interventions were conducted in a randomised fashion using a closed system (Ambu ‘E’ valve) with a tight-fitting mask: 20 minutes of normoxia (21% O2), 20 minutes of hyperoxia (40% O2), and 20 minutes of hypoxia (12% O2).

To avoid the contaminating effects of hypocapnia-mediated vasoconstriction, all interventions were conducted in eucapnia by continuously monitoring and adjusting end-tidal CO2. The cerebrovascular resistance (CVR) was calculated as mean arterial pressure/MCAv, and the arterial blood content of oxygen (CaO2) was calculated as Hgb x SaO2 + PaO2 x 0.01, where Hgb is the haemoglobin concentration, and SaO2 and PaO2 is the arterial oxygen saturation and partial pressure of oxygen, respectively. COVR was subsequently calculated as the change in CVR per mmol change in CaO2 by linear regression.

The pro-inflammatory cytokine tumour necrosis factor alpha (TNF) was measured in arterial plasma by use of a Multiplex assay (Luminex). LPS induced an immense systemic inflammatory response with fever, flu-like symptoms, neutrocytosis, and a 34 (mean, SD 12)-fold increase in the circulating levels of TNF (all P < 0.001). However, LPS infusion per se neither influenced CaO2, cortical oxygenation (both NS), nor COVR (Figure 1).

Although LPS-infusion induced a marked systemic inflammatory response, it did not appear to affect COVR. Our data suggest that COVR is intact, at least in the early stages of sepsis. Future studies should address other potential mechanisms of sepsis-associated brain dysfunction, such as impaired cerebral blood flow autoregulation and CO2 reactivity.

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**Figure 1. Effects of a four-hour lipopolysaccharide (LPS) infusion on cerebral oxygen vasoreactivity (COVR) in ten healthy humans (mean ± SEM). AU: arbitrary units.**


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Exercising Arterial Oxygenation Oxidative Stress and NO: Impact on the Regulation of Blood Coagulation in Man

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Patients with peripheral arterial occlusive disease undertake prescribed exercise experiencing hypoxaemia with periods of ischaemia-reperfusion. Hence this population regularly encounters sinusoidal changes in arterial oxygen tension. Whilst hypoxia and exercise have been independently associated with activation of blood coagulation in humans (Weiss et al, 1998; 2002) our laboratory recently reported the significance of plasma volume correction for the interpretive analysis of haemostasis (Fall et al, 2011). We have also documented redox regulation of blood coagulation following exercise (New et al, 2011). The additional effect of hyperoxic exercise on the coagulation cascade is not known. The current study tested the hypothesis that an acute bout of mild hypoxic and hyperoxic exercise would not lead to a hypercoagulable state.

9 males, MAP = 106 ± 5 mmHg were studied for 2-hours following 30-minutes of cycle exercise at 75% maximal oxygen consumption in hypoxia (16% O2), normoxia (21% O2) and hyperoxia (50% O2). Subjects were followed post-exercise for 2-hours on return back to normoxia. Echocardiography assessed cardiac output (Q) determined systemic vascular resistance (SVR) [MAP/Q] and vascular conductance (SVC) [Q/MAP]. Blood was sampled from an antecubital vein pre-, immediately post-, 1-hour (P1) and 2-hours post- (P2) exercise and corrected for haemoconcentration/dilution. Plasma was assayed for fibrinogen, international normalized ratio (INR), activated partial thromboplastin time (aPTT), prothrombin time (PT) parameters utilising coagulometer analysis. Plasma nitrate and nitrite (NOx) was determined fluorometrically and selective antioxidants by HPLC. Following confirmation of distribution normality using Shapiro-Wilk W tests, data were analysed using repeated measures ANOVA and Bonferroni corrected paired samples t-tests.

Hyperoxic exercise significantly attenuated the reductions in SVR and MAP (P<0.05; paired t-test) compared to normoxic and hypoxic exercise across the post-exercise period. Fibrinogen, INR, aPTT, aPTTr, PT, RSNO and NOx were unmodified by the acute exercise bout or oxygen tension. Exercise per se increased LOOH concentration by P1 (P<0.05; paired t-test) and LOOH concentration post exercise (A) inversely correlated with ΔINR (r = -0.50; P<0.01; pearson correlation).

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A Conserved Tryptophan at the Membrane-Water Interface Acts as a Gatekeeper for Kir6.2/SUR1 Channels and Causes Neonatal Diabetes when Mutated

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We identified a novel heterozygous mutation, W68R, in the Kir6.2 subunit of the ATP-sensitive potassium (KATP) channel in a patient with remitting relapsing neonatal diabetes. This tryptophan is absolutely conserved in mammalian Kir channels. The functional effects of mutations at residue 68 of Kir6.2 were studied by heterologous expression in Xenopus oocytes and by homology modelling.

We found the Kir6.2-W68R mutation increased the whole-cell KATP current, and reduced MgATP inhibition in both the homo-meric (hom) and heterozygous (het) state: IC50 were 142±12μM (S.E., n=13) for homW68R, 41±7μM for hetW68R and 16±1μM (n=26) for wild-type (WT) channels. Substitution of a glutamate residue (homW68E) produced an identical effect: IC50~153±18 μM (n=7). Replacement with tyrosine (Y) rendered the KATP channel almost completely insensitive to ATP block. The effect of mutations at W68 was not charge or size dependent, the order of potency for ATP inhibition being W>M>L>R=E>K>A>C>F=Y. Mutation of W68 dramatically increased the unliganded channel open probability (P o(0)) which was ~0.8 for W68A, W68F and W68Y channels versus ~0.4 for WT channels. The PIP2 affinity, as judged by the sensitivity of channel to inhibition by neomycin, was also affected, but did not explain the reduced ATP sensitivity: IC50 were WT, 129±15μM (n=8); W68R, 11.3±2.1μM (n=4); W68E, 8.2±0.9μM (n=4); W68F, 148±32μM (n=5); W68Y; <25% block at 10 μM neomycin.

In different Kir crystal structures the residue corresponding to W68 adopts two distinct positions. In most Kir, the tryptophan is absolutely conserved in mammalian Kir channels but did not explain the reduced ATP sensitivity: IC50 were WT, 129±15μM (n=8); W68R, 11.3±2.1μM (n=4); W68E, 8.2±0.9μM (n=4); W68F, 148±32μM (n=5); W68Y; <25% block at 10 μM neomycin.

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PC218

Involvement of STIM and ORAI in the Differentiation of Human Preadipocytes

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Modulation of cellular calcium concentration by hormones or neurotransmitters through Ca2+ pumps, channels and transporters is central to the physiological and pathophysiological control of cellular processes, such as vesicular trafficking, gene expression, apoptosis and necrosis. In human preadipocytes, store-operated calcium (SOC) channels have been demonstrated as a major calcium influx component. Here our aim is to detect the existence of Stim and Orai mRNAs and proteins in preadipocytes and also determine their roles in preadipocytes differentiation.

Subcutaneous adipose tissues were collected from patients undergoing bariatric surgery after ethic approval and informed consent. Preadipocytes were isolated by collagenase digestion and cultured. Cells were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 5% fetal bovine serum. Cell differentiation was induced by 3-isobutyl-1-methylxanthine, hydrocortisone, insulin and indomethacin. Immunostaining, real-time PCR, oil-red staining and Ca2+ imaging of the preadipocytes samples were performed over a 14 days’ time.

The RT-PCR and immunostaining showed the presence Stim1, Stim2, Orai1, Orai2 and Orai3 in the preadipocytes. The mRNA expression of Stim1, Stim2, Orai1 and Orai3 was significantly increased after the induction of cell differentiation for 14 days, but a small decrease for Orai2. Thapsigargin induced store-operated Ca2+ influx in both differentiated and non-differentiated preadipocytes, and the Ca2+ influx in the store-depleted preadipocytes was higher than that in cells without store-depletion.

Our results showed the presence of Stim and Orai genes and proteins in human preadipocytes. These genes are up-regulated after cell differentiation, which suggests the involvement of store-operated channel molecules in preadipocyte differentiation and obesity development.


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PC219

Metabolomics by NMR of mice blood and urine after an exhaustive exercise at individual oxidative velocity

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Metabolomic associates a quantitative analysis method of metabolites contained in biological samples to a multivariate statistical analysis and allows either to establish metabolic profiles as in pathology (obesity, muscular dystrophy, diabetes), or to identify biological markers.

The objective of this study is the investigation by 1H NMR spectroscopy of metabolome in mice having performed an exhaustive exercise either at the critical velocity (Vc) which delineate the solicitation of the aerobic metabolism or the peak velocity (Vp).

Mice were divided into 3 groups: 10 "control" mice, 7 Vc mice exercising at Vc and 13 Vp mice exercising at Vp, 2h before sacrifice. The total of the exercise was implemented for each mouse. Before sacrifice, blood retrobulbar sampling was performed from anesthetized mice (with an intra-peritoneal injection of pentobarbital (70mg.kg-1) and killed by cervical dislocation). Blood was deproteinized with trichloroacetic acid. The urine was collected, either in the cage before sacrifice, or directly from the bladder after sacrifice. The 1D proton spectra of blood or urine samples were obtained using a Varian spectrometer at 500 MHz. The spectra were divided into 0.001 ppm bins. Their intensities were analyzed by partial least square analysis (PLS) using the Simca-P + software according to their group or to the duration of exercise.

Multivariate analysis of blood sample spectra could discriminate the control group from both groups Vc and Vp taken together. The spectra could not be discriminated according to the duration of the exercise.

For urine samples, a significant model PLS model could be calculated when comparing the control mice with both groups, Vc and Vpeak. The best model was a PLS model obtained according to the exercise duration with all mice. Taking into account the spectral region having the highest effect on the calculated components of the models, glucose and dimethylamine are characterizing the control mice in blood samples while in urine samples, lactate allantoin, citrate, hippurate and carnitine were the most discriminating metabolites.

Metabolic profiles assessed with NMR are highly dependent on the exercise. These results show that urine samples were more informative than blood samples and that the duration...
of the exercise is a more important parameter to influence the metabolic status than the velocity of the exercise.

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**PC220**

**Effect of hyperglycemia on perineal musculature in erectile dysfunction on streptozotocin-induced long term diabetic male rat**

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The objective of the present study is to analyze the role of perineal muscle in diabetic-induced erectile dysfunction. Male albino rat (Rattus norvegicus) were randomly divided into 2 groups (N = 12) group I (sham received citrate buffer), group II (STZ) received single i.p. injection of 60 mg/kg bw of streptozotocin (STZ) in 0.1 M citrate buffer. At the end of the experimental period (16 week) the animals were subjected to various analyses like serum hormone, erectile function, electromyogram of ischiocavernosuss (ICM) and bulbospongiosus (BSM) (performed under Ketamine 80 mg/kg b.w., i.p. anesthesia), histology and histomorphometry of both muscle and immunohistochemistry of the synaptophysis were done. The result showed significant reduction in serum hormone (p<0.01), erectile function (p<0.001), reduced action potential in both muscles and wide range of histological alteration (thinning and crowding of muscle fiber) was observed in diabetic rats compared to control. Histomorphometrical study showed significant reduction in the diameter (0.001), volume (0.01) and numerical density (0.01) of the fiber in both muscles of diabetic rat compared to control. Immunolocalization of synaptophysis protein was found to be significantly (0.001) reduced in diabetic animal when compared to control. From the present study it was concluded that degenerative changes in IC and BS may play a major role in erectile dysfunction in diabetic condition. This might be due to diabetic induced postsynaptic neuronal degeneration respected with impaired motor action of the muscle. Thus while studying the treatment strategies for erectile dysfunction due consideration should be given to the integrity of the perineal musculature.

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**PC221**

**Effects of the CB1 cannabinoid receptor on fluid intake and hormone secretion induced by water restriction**

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The endocannabinoid system participates in several homeostatic mechanisms, being the CB1 cannabinoid receptor (CB1R) the main subtype involved in the regulation of body fluid homeostasis. This study evaluated the effects of pretreatment with AM251, a CB1R antagonist, on fluid intake and hormone secretion in response to water restriction (WR). Male Wistar rats (n=5-13) had water but not food removed 24 hours (h) before the experiment. AM251 (3mg/Kg/1.5ml, or vehicle, 5% ethanol in 0.9% NaCl) was intraperitonially administered 30 minutes (min) before fluids were presented. The first group of animals had water and 1.8% NaCl intakes assessed in intervals for 2h after fluid presentation. In another group of animals, plasma concentrations of vasopressin (AVP), oxytocin (OT), atrial natriuretic peptide (ANP), angiotensin II (ANGII) and sodium were determined from trunk blood, which was obtained by decapitation immediately before fluids presentation. WR induced a significant increase in both water (0.34±0.26 vs. 2.79±0.77 ml/100g of body weight (b.w.), p<0.05, t=20 min) and 1.8% NaCl intake (0.01±0.01 vs 1.98±0.55 ml/100g of b.w., p<0.01, t=20 min), as well as in plasma sodium concentrations (135.28±1.40 vs 144.00±0.93 mEq/l, p<0.001). AM251 also increased OT (1.47±0.19 vs 5.18±0.61 pg/ml, p<0.001) and AVP secretion (1.35±0.12 vs 7.57±0.94 pg/ml, p<0.001), although no significant changes were observed in ANGII circulating levels after the same stimulus. On the other hand, WR significantly decreased ANP plasma concentrations (55.54±9.10 vs 17.54±4.36 pg/ml, p<0.001). Previous administration of AM251 significantly reduced 1.8% NaCl ingestion induced by WR (3.18±0.75 vs 1.58±0.47 ml/100g of b.w., p<0.01, t=60 min), but it did not alter the effects of WR on water intake. Furthermore, AM251 further increased plasma sodium concentrations in WR rats (144.00±0.93 vs 150.00±1.83 mEq/l, p<0.05). Pretreatment with AM251 potentiated OT secretion in euhydration (1.47±0.19 vs 2.92±0.51 pg/ml, p<0.05) but not in WR rats; no additional changes were produced by AM251 in AVP secretion induced by WR. On the other hand, previous administration of AM251 significantly enhanced ANGII secretion induced by WR (18.68±3.91 vs 240.16±34.40 pg/ml, p<0.001) and induced a further decrease in ANP secretion in euhydration (55.54±9.10 vs 27.53±4.18 pg/ml, p<0.001) but not in WR rats. These data suggest that the CB1R: 1) participates in the control of sodium intake; 2) modulates neurohypophyseal hormone secretion; 3) regulates the secretion of peripheral hormones (ANGII and ANP).

This work is supported by Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (Grant #2010/02748-4).

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**PC222**

**The beneficial effect of GLP-1 treatment in type 1 diabetic rats**

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Diabetes mellitus is a major metabolic disorder resulting in numerous long-term complications. Factors that can reduce these complications and increase the ability of pancreatic islets to secrete insulin are of tremendous benefit to diabetic patients. This study investigated the beneficial effects of glucagon-like peptide-1 (GLP-1) on streptozotocin (STZ)-induced type 1 diabetic rats compared to healthy, age-matched
controls. The study employed four groups of rats (2 age-matched controls; 2 rendered diabetic using a single intraperitoneal (i.p.) injection of STZ (60 mg kg⁻¹)). Two groups of rats (either control or diabetic) were untreated while rats from the other two groups were given GLP-1 (50 nmol/kg body weight i.p.) for 10 weeks. The project had the relevant ethical clearance from the Ethics Committees of both UCLAN and UAE University to undertake the study. GLP-1 can evoke significant (Student’s t-test, p<0.05) increases (7.4±0.4 g) for GLP-1-treated controls vs. 5.9±0.3 for untreated controls compared to 4.8±0.1 for GLP-1-treated diabetic vs. 3.5±0.2 for untreated diabetic rats) in serum insulin (μU/ml-1) in both normal and diabetic-treated rats compared to untreated animals. Immunohistochemistry shows significant increases (in percentages) in (a) the number of insulin-positive cells (84.9±1.1 for GLP-1-treated vs. 80.2±1.3 for untreated controls; p< 0.05, compared to 32.0±1.7 for GLP-1-treated diabetic vs. 7.9±0.7 for untreated diabetic rats; p<0.001), (b) catalase-positive cells (98.0±0.8 for GLP-1-treated controls vs. 95.0±1.0 for untreated controls compared to 87.2±1.3 for GLP-1-treated diabetic vs. 66.3±2.9 for untreated diabetic rats; p<0.001) and (c) glutathione reductase-positive cells (96.0±1.3 for GLP-1-treated controls vs. 93.0±1.1 for untreated controls compared to 88.4±2.0 for GLP-1-treated diabetic vs. 66.2±3.7 for untreated diabetic rats; p<0.001). Gene expression studies show significant increases (relative quantification (RQ)) in the levels of mRNAs for (a) pro-collagen α1(I) mRNA in pancreatic islet cells of diabetic rats. GLP-1 seems to repair mitochondrial function in cultured myocytes. Chronic exercise in the absence of ovarian hormones enhances stress-induced gastric and colonic oxidative damage by a glucocorticoid receptor-dependent mechanism

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Multiple studies have shown that physical exercise, which may be a stressful stimulus in activating the hypothalamic–pituitary–gonadal axis and elevating circulating glucocorticoids (GC), is also a strong inducer of lipid peroxidation that is likely to occur after moderate to exhaustive exercise. Postmenopausal women with higher fitness levels were shown to have higher antioxidant enzyme activity and lower levels of lipid peroxidation (Pialoux et al. 2009), but their responses to stressful events are not well studied. In the present study, the effect of moderate exercise (30 min/day 5 days/week swimming for 9 weeks) on the gastric and colonic tissues of female Sprague-Dawley rats (2-3-month-old; n=80) with intact ovaries or ovariectomy (OVX) that were exposed to a painless psychological stress (30 min) for 3 days was investigated. OVX or sham operation was conducted under ketamine and chlorpromazine (100 and 2 mg/kg, respectively, i.p.) anesthesia and rats were injected with either saline or glucocorticoid receptor antagonist (RU486, 10 mg/kg; i.p.) during stress exposure. At the end of the last stress procedure, rats were decapitated and trunk blood samples were obtained for the measurement of serum levels of cortisol, IL-1β and TNF-α, while malondialdehyde (MDA), glutathione (GSH) levels and myeloperoxidase (MPO) activity were studied in the gastric and colonic tissue samples. Values are means ± S.E.M. compared by ANOVA. Although the applied stress did not result in significant elevations in serum cortisol levels in either sham or OVX rats, GC blockade elevated serum cortisol levels (p<0.01). In sedentary OVX rats, TNF-α and IL-1β levels were increased in response to stress with respect to control levels (p<0.05) and they were reduced in the trained OVX rats (p<0.01). Colonic and gastric MDA levels, indicating lipid peroxidation, were not changed in sham-operated sedentary groups with stress exposure, but they were significantly increased in the trained OVX rats (p<0.001), while RU486 treatment further enhanced MDA levels in the trained OVX groups (p<0.01). Exposure to stress in neither exercised nor sedentary groups with intact ovaries did not alter MPO activities in the gastric or colonic tissues, while increased gastric and colonic MPO activities in the exercised rats with OVX were increased more by GC blockade (p<0.001). Antioxidant GSH levels in gastric and colonic tissues were increased in the exercised rats with OVX (p<0.01), but not in the sedentary rats. However, RU486 treatment abolished exercise-induced elevations in GSH levels (p<0.01). In conclusion, the results demonstrate that in the absence of ovarian hormones, moderate exercise depresses stress-induced cytokine response and improves antioxidant status, but augments lipid peroxidation by a glucocorticoid receptor-dependent mechanism.

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**PC223**

**Chronic exercise in the absence of ovarian hormones enhances stress-induced gastric and colonic oxidative damage by a glucocorticoid receptor-dependent mechanism**

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Multiple studies have shown that physical exercise, which may be a stressful stimulus in activating the hypothalamic–pituitary–gonadal axis and elevating circulating glucocorticoids (GC), is also a strong inducer of lipid peroxidation that is likely to occur after moderate to exhaustive exercise. Postmenopausal women with higher fitness levels were shown to have higher antioxidant enzyme activity and lower levels of lipid peroxidation (Pialoux et al. 2009), but their responses to stressful events are not well studied. In the present study, the effect of moderate exercise (30 min/day 5 days/week swimming for 9 weeks) on the gastric and colonic tissues of female Sprague-Dawley rats (2-3-month-old; n=80) with intact ovaries or ovariectomy (OVX) that were exposed to a painless psychological stress (30 min) for 3 days was investigated. OVX or sham operation was conducted under ketamine and chlorpromazine (100 and 2 mg/kg, respectively, i.p.) anesthesia and rats were injected with either saline or glucocorticoid receptor antagonist (RU486, 10 mg/kg; i.p.) during stress exposure. At the end of the last stress procedure, rats were decapitated and trunk blood samples were obtained for the measurement of serum levels of cortisol, IL-1β and TNF-α, while malondialdehyde (MDA), glutathione (GSH) levels and myeloperoxidase (MPO) activity were studied in the gastric and colonic tissue samples. Values are means ± S.E.M. compared by ANOVA. Although the applied stress did not result in significant elevations in serum cortisol levels in either sham or OVX rats, GC blockade elevated serum cortisol levels (p<0.01). In sedentary OVX rats, TNF-α and IL-1β levels were increased in response to stress with respect to control levels (p<0.05) and they were reduced in the trained OVX rats (p<0.01). Colonic and gastric MDA levels, indicating lipid peroxidation, were not changed in sham-operated sedentary groups with stress exposure, but they were significantly increased in the trained OVX rats (p<0.001), while RU486 treatment further enhanced MDA levels in the trained OVX groups (p<0.01). Exposure to stress in neither exercised nor sedentary groups with intact ovaries did not alter MPO activities in the gastric or colonic tissues, while increased gastric and colonic MPO activities in the exercised rats with OVX were increased more by GC blockade (p<0.001). Antioxidant GSH levels in gastric and colonic tissues were increased in the exercised rats with OVX (p<0.01), but not in the sedentary rats. However, RU486 treatment abolished exercise-induced elevations in GSH levels (p<0.01). In conclusion, the results demonstrate that in the absence of ovarian hormones, moderate exercise depresses stress-induced cytokine response and improves antioxidant status, but augments lipid peroxidation by a glucocorticoid receptor-dependent mechanism.

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**PC224**

**Effects of chronic insulin and saturated fatty acid exposure on mitochondrial function in cultured myocytes**

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Mitochondrial dysfunction has been associated with insulin resistance, obesity and diabetes. However, whether mitochondrial dysfunction is the consequence or cause of insulin resistance is controversial. Insulin signalling has been shown to regulate expression of mitochondrial genes. Here we examined the independent contributions of impaired insulin signalling and saturated fatty acid exposure on mitochondrial function and gene expression in cultured myocytes. Chronic insulin treatment of myotubes resulted in insulin resistance, with reduced signalling through Phosphatidylinositol 3-Kinase/AKT signalling pathway. Insulin-resistant myocytes exhibited lower mitochondrial membrane potential and ROS production but comparable ATP production to control cells. The activity of the mitochondrial marker citrate synthase was impaired in insulin-resistant myocytes, but not in control cells. These findings suggest that chronic insulin and fatty acid exposure may contribute to mitochondrial dysfunction in insulin-resistant myocytes.
increased in insulin-resistant cells compared to controls. The expression of a number of mitochondrial genes including COX1, COX2, COX4, ATP synthase, Tαm, NRF1, Mitofusin 2, and porin were also largely unaffected by chronic insulin exposure. However, the transcriptional co-activator PGC1α, and uncoupling proteins, UCP2 and UCP3 were significantly reduced in this insulin resistant cell model. In contrast, treatment of cells with saturated fatty acid reduced mitochondrial membrane potential but significantly increased ROS production, and increased the mRNA expression of UCP2, UCP3 and ATP synthase. These cells also displayed lower levels PGC1α and PGC1β gene expression compared to controls. Taken together these results suggest that chronic hyperinsulinemia causes insulin resistance but not mitochondrial dysfunction. However, insulin fails to protect against fatty acid-induced mitochondrial dysfunction.

This work was supported by a research grant from the European Commission Framework 7 (HEALTH-F4-2008-223450)

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PC225

Low-sodium diet induces endocrine and hydroelectrolytic changes in male rats
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The salt intake is a behavior implicated in the integrative regulation of extracellular volume and blood pressure and is directly influenced by changes in sodium homeostasis. The sodium dietary restriction is an interesting noninvasive model to study the sodium appetite. Our aim was to investigate the integrative control of salt and water intake balance after dietary sodium restriction. To this, Wistar male rats receive normal (1% NaCl) or low- (<0.05% NaCl) Na+ diet during four days. Food intake did not differ between normal and low-Na+ diet groups. However, we observed a reduced body weight gain during the four days of low-Na+ diet (20.0 ± 1.9 g/100g vs. 36.2 ± 3.9 g/100g, p < 0.01) associated with a decrease in water intake (9.6 ± 0.8 mL vs. 13.1 ± 0.5 mL, p < 0.05) and a decrease in the extracellular volume as demonstrated by increase in hematocrit (42.8 ± 0.7 % vs. 38.8 ± 0.6 %, p < 0.01) and plasma proteins (7.0 ± 0.1 mg/dL vs. 5.7 ± 0.1 mg/dL, p < 0.001). Additionally, the low-Na+ diet induces a decrease in urine volume (5.4 ± 0.6 mL vs. 7.6 ± 0.5 mL, p < 0.05) and sodium excretion (0.26 ± 0.01 mEq/100g/day vs. 1.66 ± 0.07 mEq/100g/day, p < 0.01). We observed a discrete decreased arterial pressure (101 ± 1.5 mmHg vs. 105 ± 1.1 mmHg, p < 0.05) without effect in heart rate. There was no effect of low-Na+ diet on plasma atrial natriuretic peptide and vasopressin concentrations. An increase in plasma angiotensin II (119 ± 12 pg/mL vs. 39 ± 11 pg/mL, p < 0.001) and decrease in plasma oxytocin (0.60 ± 0.04 pg/mL vs. 0.99 ± 0.12 pg/mL, p < 0.05) concentrations after low-Na+ diet was observed. These animals also presented an increase in 1.8% NaCl intake at 30 min (0.44 ± 0.05 mL vs. 0.06 ± 0.02 mL, p < 0.05) to 5 hours (1.65 ± 0.13 mL vs. 0.57 ± 0.05 mL, p < 0.001) after four days of low-Na+ diet. Associated to this, low-Na+ diet group also presents an sodium intake-dependent increase in water intake at 1 hour (0.99 ± 0.09 mL vs. 0.49 ± 0.12 mL, p < 0.05) to 5 hours (2.86 ± 0.13 mL vs. 1.64 ± 0.21 mL, p < 0.001). Regarding the sodium appetite, the low-Na+ diet induce an important increase in sodium preference at 30 min (31.4 ± 3.5 % vs. 12.9 ± 4.8 %, p < 0.001) to 5 hours (34.1 ± 1.9 % vs. 22.1 ± 1.9 %, p < 0.01). Our results indicate that sodium appetite control and the strong increase in sodium preference after sodium restriction is dependent on a variety of factors, that could be included the activation of sodium appetite signals (volume/ baroreceptors and peripheral Ang II) and inhibition of sodium satiety signals, as oxytocin.

Financial Support: CNPq/FAPESP

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PC226

HIF-1α mediates prostate hyperplasia under chronic inflammation
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Benign prostatic hyperplasia (BPH) commonly occurs in aged men, and concurrently with chronic prostatitis. Although inflammatory signs are frequently found in BPH specimens, it is unclear if inflammation initiates or aggravates BPH. Recently, we reported that HIF-1α is involved in testosterone-induced prostate hyperplasia and that the HIF-1α inhibition blocks the disease progress. Given that the HIF-1α synthesis is facilitated by proinflammatory cytokines, we here tested the possibility that the cytokines are secreted from immune cells infiltrating into the prostate and enlarges the prostate by upregulating HIF-1α. All animal procedures were performed in accord with the Seoul National University Laboratory Animal Maintenance Manual. To examine whether the prostate becomes hyperplastic under inflammatory conditions, we treated rat prostates with LPS in vivo. We injected 150 μl of LPS (0.1 μg/g body weight) or vehicle (DMSO) into the prostates of rats which had been anesthetized by isoflurane (2% for 10 min). The same procedure was done again 7 days later. On the seventh day after the second injection, each rat was killed by CO2 asphyxiation, and the prostate was excised. We found that HIF-1α was upregulated in the epithelial cells of LPS-treated prostates. Moreover, cell proliferation was promoted, and cell death was demoted in the prostates. To understand the mechanism underlying the LPS-induced induction of HIF-1α, we directly treated PC3 (human BPH cell line) or LNCaP (prostate cancer cell line) cells with LPS (5 μg/ml), but HIF-1α was not significantly induced. Interestingly, HIF-1α and VEGF expressions were highly induced in prostate cells which had been cultured in the conditioned media obtained from LPS (0.1 μg/ml)-activated THP-1 macrophages. We further studied to identify the cytokines responsible for HIF-1α induction, and also to clarify the mechanism by which HIF-1α induces prostate overgrowth under inflammatory circumstances.


Reactive oxygen species (ROS) from cytochrome P450 2C9 mediate Ca²⁺ release from endothelial stores during endothelial derived hyperpolarising factor (EDHF) responses

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EDHF is the endothelium-dependent but nitric oxide and prostacyclin-independent vasodilatation pathway and has been characterised as requiring KC₃.1 and KC₃.2 channel activation as well as functional myoendothelial gap junctions. H₂O₂ has also been implicated in this EDHF response, but its role has yet to be elucidated. To investigate this the cremaster muscle circulation of freshly killed Wistar rats was perfused with a Krebs buffer solution containing albumin (1 g.l⁻¹) and Fura-PE3 AM (10 μM) for 60 minutes, which led to selective loading of the Ca²⁺ indicator into the endothelium. The preparation was placed on the modified stage of an intravital microscope to measure vessel diameter, and endothelial [Ca²⁺]i was estimated from the 360/380nm excitation ratio, emission > 510nm, using an extended ISIS camera. The significance of changes was assessed by paired t test. The preparation was routinely superfused with phenylephrine (30 nM), L-NAME (300 μM) and indomethacin (3μM). This caused a 36.8 ± 3.2%, n=54 (mean ± SEM) constriction of the maximum diameter. Relaxation was measured as the % decrease in constriction. Addition of carbachol (10μM) resulted in 74.5 ± 2.0%, n=69. The EDHF mediated relaxation and endothelial [Ca²⁺]i increase in response to carbachol was substantially reduced by including a ROS scavenging combination of superoxide dismutase and catalase (100U.ml⁻¹ each; relaxation from 74.9 ± 8.9% to 37.5 ± 13.5%, ratio from 19.3 ± 3.3% to 5.3 ± 1.7%, n=6; p<0.05). The possibility that ROS are important for Ca²⁺ release from stores was examined by applying EGTA in Ca²⁺-free solutions. Carbachol application then resulted in transient Fura ratio increase that was reduced by SOD and catalase (from 20.5 ± 2.8% to 10.4 ± 1.5% n=5; p<0.01). The phospholipase C inhibitor U73122 (3μM) reduced the EDHF response to carbachol (relaxation 74.9 ± 2.8% to 10.6 ± 2.4%, ratio 22.9 ± 2.5% to 3.2 ± 0.6%, n=5; p<0.05). The KC₃.1 and KC₃.2 channel activator NS309 (10μM) caused a comparable relaxation to carbachol (65.6 ± 2.2%, n=5) that was not blocked by SOD and catalase (relaxation 64.3 ± 2.0%, n=5) suggesting the ROS work before this step in the pathway. The selective CYP 2C9 inhibitor sulphanfenazole (10μM) reduced relaxation (from 80.2±3.6% to 23.7 ± 2.6%, n=5; p<0.05) and the endothelial [Ca²⁺]i increase (from 23.1 ± 2.3% to 8.6 ± 1.2%, n=5; p<0.05). In the Ca²⁺-free preparation sulphanfenazole also inhibited the Ca²⁺ release from stores (ratio from 17.0 ± 2.0% to 7.7 ± 0.8%, n=5; p<0.05). These data suggest that ROS produced by arachidonic acid metabolism via CYP 2C9 promote EDHF mediated relaxation mainly by enhancing the release of Ca²⁺ from endothelial stores.

Funded by the British Heart Foundation, PhD FS/08/057/25816.

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PC229
Effects of hyperbaric oxygen therapy on reactivity of rat aortic rings to angiotensin II and angiotensin-(1-7)
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INTRODUCTION: Hyperbaric oxygen therapy (HBOT) has various effects on perfusion and vascular function and it has been hypothesized that HBOT may change vascular sensitivity to different dilators and constrictors [1]. Angiotensin-(1-7) (ANG-(1-7)) has been demonstrated to have dilating activity, in contrast to angiotensin II (ANG II) [2]; both peptides having complex mechanisms of action. The aim of our study was to assess whether there are effects of HBOT on vascular reactivity to ANG II and ANG-(1-7).

METHODS: Thoracic aortic ring preparations from Sprague-Dawley rats, divided into an HBOT and a control group, were used to test responses to ANG II, ANG II+ ANG-(1-7) or ANG-(1-7) after preconstriction with noradrenaline. HBOT rats were treated in a hyperbaric chamber with 100% O2(2 bar) 2 hours/day for 4 consecutive days. After intactness of endothelium was tested with acetylcholine, each ring was subjected to maximal contraction (using 60μM KCl + 0.1μM noradrenaline), and after washing/equilibration treated with either 1 μM ANG II (n[HBOT]=17; n[control]=16), 1 μM ANG II+1 μM ANG-(1-7) (n[HBOT]=14; n[control]=17), or noradrenaline 0.1 μM for 5 minutes, after which 1 μM ANG-(1-7) was added and the ring tension read after 3 minutes (n[HBOT]=12; n[control]=17). The peak contraction force after ANG II and ANG II+ ANG-(1-7) was expressed as percentage of maximal contraction, the effect of ANG-(1-7) addition was expressed as percentage of precontraction decrease after 3 minutes.

RESULTS: Mean percentage of maximal contraction for ANGII was 21%±11 (HBOT) and 20%±9 (control) and was similar between groups (Mann-Whitney U test, P=1,000). The mean percentage for ANGII + ANG-(1-7) was 15%±10 (HBOT) and 20%±9 (control); it tended to be decreased in the HBOT group, but without statistical significance (Mann-Whitney U test, P=0,054). There was a statistical significance when the mean percentage of maximal contraction of ANGII (HBOT) was compared to ANGII + ANG-(1-7) in the HBOT group (P=0,029[Mann-Whitney U]), without such a difference within the control group (P=0,953, t-test). Mean percentage of noradrenaline precontraction decrease with ANG-(1-7) was 10%±5 (control) and 19%±11 (HBOT) - significantly different between groups (t-test, P=0,017).

CONCLUSION: There was no change in reactivity to ANG II after HBOT. There may be an increase of reactivity to ANG-(1-7) after preconstriction with noradrenaline, but since there was no significant effect of HBOT on the ANG II + ANG-(1-7) peak compared to control, at least at 1 μM concentrations, further studies are needed to conclusively interpret these results. Interestingly, there is a difference between the peak of ANGII in the HBOT group and ANGII + ANG-(1-7) in the HBOT group, whereas this difference was not present in the control group, suggesting an influence of HBOT on vascular reactivity.

PC230
Leucocyte infiltration in experimental warm hepatic ischemia reperfusion model; effect of ischemic pre and post conditioning strategies and implications of adhesion molecules
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Aim: Few studies are available regarding the role of ischemic postconditioning (IPO) in the prevention of hepatic Ischemia-reperfusion (I/R) injury. The present study wonders if IPO can attenuate leucocyte infiltration in I/R model. We also aimed to compare such effect of IPO with that produced by ischemic preconditioning (IPC) and to study their relation to vascular cell adhesion molecule-1 (VCAM-1) and E selectin adhesion molecules.

Methods: 40 male Wistar rats (anesthetized by intraperitoneal injection of pentobarbital 40 mg/Kg body weight) were randomly divided into 4 groups (n=10); group I (control group, subjected to sham laparotomy). Group II (I/R, subjected to ischemia of the left lateral and median lobes using atraumatic clamp for 60 minutes to induce partial hepatic warm ischemia followed by removal of the clamp and reperfusion for 120 minutes). Group III (IPC, exposed to 10 minutes ischemia and 15 minutes reperfusion prior to I/R); and Group IV (IPO, underwent 3 brief cycles of ischemia separated by reperfusion with 30 seconds each at the onset of reperfusion following the 60 minutes ischemia). The study protocol was approved by the ethical committee, Kasr-Al Aini Faculty of Medicine, Cairo University.

Colorimetric assessment of serum ALT and albumin as indicators of hepatocellular injury was carried out. VCAM-1 and E selectin levels were determined in serum and ischemic liver tissue homogenate using ELISA. The myeloperoxidase (MPO)
activity which is used as an index of leucocyte accumulation was assayed spectrophotometrically. Statistical analysis was made using one way analysis of variance; data are presented as mean ± standard deviation and association of variables was calculated using Pearson correlation (SPSS version 12). P values < 0.05 were considered as statistically significant.

Results: IPO attenuated the liver functional damage following I/R by reducing serum ALT (P < 0.05), and increasing albumin level which didn’t reach significance. IPO also decreased liver MPO (P < 0.01 compared to I/R group), although this reduction was less marked when compared with IPC group (P < 0.001). IPO significantly lowered the elevation in liver VCAM-1 and E selectins produced by I/R (P < 0.01 for both). Correlation results demonstrated a positive correlation between liver VCAM-1 and MPO (r = 0.723, P < 0.05) and a negative correlation between soluble fraction of E selectin and MPO (r = -0.687, P < 0.05) in the I/R group. Conclusion Our results revealed that IPO could contribute to liver protection against I/R injury by attenuating leucocyte infiltration and decreasing VCAM-1 and E selectin levels in the liver. We also postulate that soluble E selectin can be used as a serum marker to anticipate the degree of leucocyte infiltration in hepatic I/R injury.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC231**

**SRPK1 inhibition significantly reduces laser-induced choroidal neovascularisation (CNV) and intravitreal neovascularisation (IVNV) in an Oxygen Induced Retinopathy (OIR) model of Retinopathy of Prematurity (ROP) in rats**

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Vascular endothelial growth factor (VEGF) induces abnormal growth of retinal blood vessels in Retinopathy of Prematurity (ROP), a leading cause of blindness in children. Serine rich protein kinase 1 (SRPK1) has been identified as a target in controlling the splicing of VEGF(1). The VEGF gene is alternatively spliced into two sister families of isoforms, angiogenic VEGF<sub>AA</sub> and anti-angiogenic, VEGF<sub>BB</sub>b. Both VEGF<sub>AA</sub> and VEGF<sub>BB</sub>b isoforms are expressed in the normal retina. SRPK1 phospho-rulates and induces nuclear localization of alternative splice factor/splice factor 2 (ASF/SF2), a promoter of VEGF<sub>AA</sub> splice site selection, thus upregulating pro-angiogenic VEGF production. We investigated whether an inhibitor of SRPK1 (2, SRPIN340(2) could alter VEGF splicing to promote VEGF<sub>BB</sub>b isoforms and inhibit pathological neovascularisation in models of eye disease.

Newborn rat pups to repeated cycles of 24 h of 50% oxygen alternating with 24h of 10% oxygen (50/10 OIR model) to cause a condition similar to human ROP. At post-natal day (P) 12 pups were anaesthetized by intraperitoneal (IP) injection of medetomidine (0.5mg/kg) and ketamine (50mg/kg), and given a 2μl intraocular (IO) injection of 10ng/μl SRPIN340 or saline. At P14 pups were removed from the oxygen chamber and left in normoxia until P20, when they were killed and the retinas removed, and flat-mounted. Additionally SRPIN340 was tested in a laser induced CNV model in Norway Brown rats, rats were anaesthetized by an IP injection of medetomidine (0.5mg/kg) and ketamine (50mg/kg). Following laser photocoagulation rats received IO injections of 25ng SRPIN340 in the ipsilateral eye and saline in the contralateral eye. 7 days later the rats were anaesthetized as above, and the injections repeated. 14 days after laser photocoagulation animals were killed by inhalation of isoflurane, and choroids dissected and flat mounted. Retinas or choroids were isoeinatin stained, and IVNV or CNV was determined by area of pathology (Imagej). SRPIN340 (p<0.05, students paired t-test) significantly reduced the IVNV area from 1.04±0.15% of the retina in control eyes (p>0.05) to 0.74±0.13% of total retina and significantly reduced CNV area (p<0.05, students paired t-test) in treated eyes (9916±2159μm²) compared to matched controls (18027±1732μm²). These results indicate that targeting SRPK1, which has been shown to switch splicing from pro to anti-angiogenic splice variants of VEGF, has the potential to prevent VEGF mediated pathological blood vessel growth in vivo. This approach would maintain the production of cyto-protective VEGF<sub>BB</sub>b isoforms compared with current anti-VEGF therapeutics that target all VEGF isoforms.


Supported by Fight for Sight Hans and Gertrude Hirsch Award, and the Skin Cancer Research Fund.

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**PC233**

**The investigation of a role for TRPV1 at a remote site in sepsis**

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Little is known about the innate protective mechanisms against the onset of sepsis the exaggerated host response to infection. There is evidence that transient receptor potential vanilloid 1 (TRPV1) exerts a protective role on lipopolysaccharide (LPS)-induced sepsis as TRPV1/-/- mice show an exacerbator of spontaneous as well as increased levels of tumour necrosis factor-α and nitric oxide in the peritoneal cavity. We have investigated whether there are differences in leukocyte-endothelial interactions and microvascular permeability in early and late sepsis in TRPV1+/+ (WT) and TRPV1/-/- (KO) mice. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the mice (25–35 g; n = 6–10) were kept in a climatically controlled environment and given food and water ad libitum. They received 11.25 × 100 endotoxin units/kg-1 i.p. or 0.9% saline i.p. and were anaesthetised with a mixture of ketamine (100mg/kg-1) and xylazine (10mg/kg-1, i.p.) at 4hrs (early sepsis) or 16hrs (late sepsis). The cremaster muscle was prepared under the microscope to quantify leucocyte rolling, adhesion and transmigration in vivo. Ex vivo permeability measurements were carried out post mortem (Schedule 1). The
aorta was cannulated, blood was flushed from the preparation and the cremaster perfused with Krebs buffer solution containing albumin and FITCalbumin (10 mg.ml-1 each). Permeability changes in response to the B1 agonist des-arg9 bradykinin (DABK; 0.01-10 μM) and B2 receptor agonist bradykinin (BK; 0.01-10 μM) were quantified in all experiments. Leukocyte rolling was assessed as no. of cells passing a point each min. Following 4hr sepsis, there was a decrease in rolling in the WT: from 18 ± 4 (mean ± sem) to 2 ± 0.5 cells.min-1; and KO mice from 32 ± 8 to 1 ± 0.25 cells.min-1 (both p < 0.05). There was no difference in rolling in sepsis between WT and KO mice. Adhesion increased by 72% WT mice (from 3 ± 11 to 11 ± 1.4 cells/100μm segment; p < 0.05) and by 44% in KO mice (from 4.5 ± 1 to 8 ± 1.2 cells/100μm segment), but again there was no difference between WT and KO mice. Transmigration increased by 2-fold in WT mice (from 3 ± 0.25 to 6 ± 0.6 cells/100 μm2) and 1.2-fold in KO mice (from 3.5 ± 0.7 to 3 ± 0.5 cells.100μm-2). There was no permeability response to DABK in any mice. The permeability responses to 10μM BK was reduced by 51% in WT and by 62% in the KO mice at 4hr sepsis, but after 18hr sepsis permeable response increased by 57% in WT and 60% in KO mice. There was no difference between the 2 groups. At present, is not clear whether or not TRPV1 receptors play a protective role in sepsis in this vascular bed as TRPV1 deletion showed very similar inflammatory responses in both WT and KO mice.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC234

Sphingosine 1-phosphate inhibits angiogenesis in human arteries

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The formation of new blood vessels by angiogenesis is a key pathophysiological process that can be activated in ischaemic conditions. The naturally occurring lipid mediator, sphingosine 1-phosphate (S1P), is released from activated platelets and at sites of thrombosis where ischaemia may result. S1P is an important regulator of the vasculature through activation of S1P receptors and could play a role in controlling new vessel formation and subsequent reperfusion of ischaemic tissues. In this study we aim to examine the effects of S1P on human arterial angiogenesis. The effects of pathological S1P concentrations on endothelial tubule formation were assessed by an ex vivo angiogenic ring model and an in vitro co-culture model. Human left internal mammary artery rings were cultured on Matrigel with standard growth factors at 37°C and incubated with vehicle or S1P (1μM). Tubule microvessel outgrowths branching from rings were counted. S1P significantly reduced mean tubule number formed at day 14 (22±3, n=14) compared to vehicle (63±9, n=20) (p=0.05, one-way ANOVA). To examine potential mechanisms, rings were pre-incubated with S1P2 receptor antagonist, JTE-013 (1μM). JTE-013 significantly reversed the decrease in tubule formation produced by S1P (n=7). As S1P2 receptors couple to the RhoA/Rho-kinase pathway, the effects of the Rho-kinase inhibitor Y27632 (10μM) were determined. Inhibition of Rho-kinase significantly reversed the S1P-induced decrease in tubule formation (44±3, n=7). A co-culture model of angiogenesis using primary cultured human fibroblasts, human coronary artery vascular smooth muscle cells and human coronary artery endothelial cells was also investigated. Co-cultures were incubated with vehicle or S1P (1μM). After 14 days, endothelial tubule formation was visualized by immunofluorescence labelling with endothelial selective anti-CD146 antibody. Fluorescent images were analysed using ImageJ software. S1P significantly inhibited tubule formation in these co-cultures by 80±7% (n=7, p<0.05, ANOVA). The signalling mechanism responsible also occurred via S1P2 and Rho/Rho-kinase signalling, as addition of JTE-013 or Y27632 significantly reversed the S1P-induced inhibition. The potential mechanisms were further investigated by examining formation of adherens junctions. Expression of VE-cadherin, an essential junctional protein, was unchanged in endothelial cells following S1P incubation. Staining with anti-VE-cadherin antibody in co-cultures, however, revealed a diffuse localization in S1P-treated co-cultures which suggests a disruption to adherens junction formation. In conclusion, S1P inhibits angiogenesis in human arteries in an S1P2 dependent manner. This S1P-induced angiostatic effect occurred through a mechanism involving a disruption of endothelial cell-cell contact.

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PC235

Quantification of tumour cell adhesion on endothelial cell monolayers

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Introduction:

Adhesion of tumour cells (TCs) to the endothelial cells (ECs) that line the circulatory system plays an important role in the process of metastasis [1-2]. A fluorescent activated cell sorting (FACS) technique has previously been used to determine the number of tumour cells that adhere to endothelial cells [3, 4]. In this study we have developed a simple assay to quantify the number of tumour cells that adhere to an endothelial cell monolayer. Haddad and colleagues (2010) have shown in their FACS analysis that after washing, about 50% of the total added TCs adhered to the EC monolayer which represented a 1 TC for 2 ECs ratio [3]. In the current study, a different but simpler approach was used to determine this ratio.

Aims and objectives:

The primary aim of this study was to develop an alternative fluorescence-based assay to quantify the adhesion of tumour cells (PC3, DU-145 and MCF7) to human vascular umbilical vein endothelial cells (HUVECs).

Methods:

Monolayers of human vascular endothelial cells (HUVEC) were activated by treating them with 10ng/mL of TNF-α. Different numbers of PC3, DU-145 and MCF7 cells (10,000-160,000) were added to each monolayer. Phase contrast and fluorescent microscopy was performed to observe tumour adhesion onto endothelial cell surfaces. Tumour cell fluorescence, as an index of cell adherence, was detected using a Fluostar Optima plate reader (BMG Labtech) with the excitation wavelength set at 485 nm and emission at 520 nm. Statistical analysis was done using a one tailed t-test.

Results:
The attachment of TCs to activated HUVEC monolayers was increased in proportion to the number of TCs added. We have also found that there is a specific ratio of TCs adhering to HUVEC monolayers which depend on the number of tumour cells added.

Conclusion:
We conclude that the number of tumour cells that adhere to HUVECs is proportionally related to the number of tumour cells used in the assay and this adherence is increased by the pre-activation of HUVECs by TNF-α.


We gratefully acknowledge the Higher Education Commission (HEC) of Pakistan and the University of Karachi who funded and supported this research.

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PC236

Mechanisms of glutamatergic regulation of capillary diameter by pericytes
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Neurovascular coupling mechanisms increase the blood flow to regions where neurons are active. Arterioles dilate to increase blood flow in response to neuronal activity, but blood flow may also be regulated by contractile pericytes on capillary walls, which dilate in response to glutamate in brain slices (Peppiatt et al., 2006). The aim of this study was to investigate the mechanisms underlying the dilation to glutamate in capillaries, and thus to better understand the contribution of pericytes to regulating blood flow. Capillaries were monitored in the molecular layer of juvenile (P12) rat cerebellar slices (200 μm) using bright field imaging. Slices were superfused with ACSF solution and oxygenated by bubbling with 95% O₂/5% CO₂, pH 7.4, 32.35°C. Pericytes were identified from their position on the outside of capillaries (defined as vessels less than 10 μm in diameter lacking a continuous layer of smooth muscle), and in some experiments by the expression of DsRed under control of the NG2 promoter.

After preconstricting pericytes with 2 μM noradrenaline (which produced a decrease in vessel diameter of 14.4±1.8%; mean+s.e.m, n=131 capillaries; Wilcoxon signed-rank test, p<0.001), exogenous glutamate (500 μM) dilated capillaries by 7.1±1.6% (n=131; Wilcoxon signed-rank test, p<0.001), and this dilation was not reduced by blocking action potentials with 1 μM TTX (Mann-Whitney U-test, p=0.054). In TTX, glutamate produced a 30.6±10.9% increase in vessel diameter (n=5; paired t-test, p<0.001) also occurred (with a latency of ~6 sec) when endogenous glutamate was released by stimulating the parallel fibres.

The glutamate-evoked dilation was blocked by inhibiting nitric oxide (NO) release with 100 μM L-nitroarginine (Mann-Whitney U-test, p=0.04). In L-nitroarginine, glutamate produced a -2.0±4.5% change in vessel diameter (n=46; Wilcoxon signed-rank test, p=0.84). Interestingly, this block of glutamate dilation by L-nitroarginine was absent if synthesis of the vasoconstricting arachidonic acid derivative 20-HETE was also prevented using 1 μM HET-0016 (Mann-Whitney U-test, p=0.006 comparing glutamate in L-nitroarginine with and without HET-0016). In L-nitroarginine plus HET-0016 glutamate produced a 24.0±8.8% increase in vessel diameter (n=10; paired t-test, p=0.048). This implies that NO acts by suppressing 20-HETE synthesis, and the continued presence of dilation when NO and 20-HETE production are blocked suggests that glutamate-evoked dilation is produced by other mechanisms, such as activity-evoked release of prostaglandin or EET derivatives of arachidonic acid.


Supported by the Fondation Leducq, Wellcome Trust, MRC, and ERC. We thank Akiko Nishiyama and Dirk Dietrich for NG2-DsRed mice.

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Apelin is a novel bioactive peptide as the endogenous ligand for the orphan G-protein-coupled receptor, APJ. Expression of both apelin and APJ are determined in various tissues such as hypothalamus, lung, uterus, ovary and the gastrointestinal tract in rat. Though some different molecular forms of apelin are turned out in tissues, apelin-13 is one of the most effective forms of apelin peptides. In this study we investigated the effect of apelin-13 on spontaneous contractions in the rat uterus in vitro.

Myometrium strips were removed from adult Wistar rats both at dioestrus and 21st day of pregnancy following decapitation at 37 oC and pH 7.4, constantly bubbled with 95% oxygen and 5% carbon dioxide. The myometrial strips were allowed to equilibrate under 1g tension and isometric contractions were measured by force displacement transducer. After diminishing of spontaneous contractions, control contractions were recorded for 10 min and increasing concentrations of apelin were added cumulatively. The amplitude and frequency under 1g tension and isometric contractions were measured by force displacement transducer. After diminishing of spontaneous contractions, control contractions were recorded for 10 min and increasing concentrations of apelin were added to the tissue bath cumulatively. The amplitude and frequencies (number of contractions for 10-minute period) of contractions were evaluated at 10-min interval before and after applications of apelin and determined as mean±SEM. One Way ANOVA was used for statistical analysis.

Apelin stimulated the spontaneous contractions in myometrial strips. The mean frequency of contractions were 1.14±0.14 (n=6), 1.00±0.00 (n=6), 1.00±0.00 (n=6), 2.57±0.30 and 4.14±0.70 (n=6) in dioestrous group and 1.17±0.17 (n=7), 1.17±0.17 (n=7), 3.33±0.80 (n=7), 4.83±0.87 (n=7) and 5.33±0.99 (n=7) in late pregnant group under control conditions and after application of 0.01μM, 0.1μM, 1μM and 10μM apelin, respectively. The increases in frequency of contractions were significant in 1μM and 10μM apelin for strips from both dioestrous (p<0.05 and p<0.001, respectively) and late pregnant rats (p<0.01, for both two concentrations). The mean peak amplitudes (gram) of contractions were 1.47±0.05 (n=6), 1.45±0.06 (n=6), 1.50±0.05 (n=6), 1.73±0.06 (n=6) and 1.91±0.10 (n=6) in dioestrous group and 2.03±0.12 (n=7), 2.03±0.12 (n=7), 2.56±0.20 (n=7), 3.01±0.27 (n=7) and 3.12±0.22 (n=7) in late pregnant rat under control conditions and after application of 0.01μM, 0.1μM, 1μM and 10μM apelin, respectively. The increase in amplitude of contractions were significant after application of 10μM apelin in strips from rats at dioestrous (p<0.01) and 1μM and 10μM apelin in late pregnant rat (p<0.05 and p<0.01, respectively).

In this study, we demonstrate for the first time that apelin has stimulatory effect on myometrial contractility in rats. Further detailed investigations are needed to clarify the exact mechanism(s) of apelin induction on myometrium contractility and the role of apelin in pregnancy and parturition processes. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
PC239

Effect of K<sub>V</sub>7 activators on chorionic plate arterial tone: A functional role for K<sub>V</sub>7 channels in the human placental vasculature?

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K<sub>V</sub>7 channels, a subfamily of voltage-gated potassium channels, may be potentially important contributors to regulation of vascular tone in human systemic arteries [1]. Previously we demonstrated that pharmacological K<sub>V</sub>7 channel manipulation modifies the reactivity of placental chorionic plate arteries (CPAs) [2]. These data support a role for K<sub>V</sub>7 channels in regulating fetoplacental resistance artery tone but the functionally important subtypes have yet to be identified. Here, we assess the responses of CPA's to structurally distinct K<sub>V</sub>7 channel openers.

Methods: Term placentas (N=14) were obtained post delivery (vaginal or caesarean section), following uncomplicated pregnancies. Biopsies were placed into ice-cold HCO<sub>3</sub>-buffered physiological salt solution (PSS). CPA's were mounted on a wire myograph, normalized at 0.9K<sub>10</sub>M (20mmHg) and equilibrated (37°C; 20 min in 5% O<sub>2</sub>/ 5% CO<sub>2</sub>/ 90% N<sub>2</sub>). Contractile responses were initially assessed with PSS containing 120mM potassium chloride (KPSS). Post wash, arteries were pre-constricted with arginine vasopressin (AVP; 10<sup>-8</sup> M) and exposed to retigabine or S-1 acrylamide (K<sub>V</sub>7 openers; 10<sup>-8</sup>-10<sup>-4</sup> M) and responses compared with diluent-treated controls (dimethylsulphoxide DMSO; maximum final concentration 1%). Experiments were repeated following pre-constriction with 80mM KPSS.

Results: Baseline arterial diameters were 326±17μm (mean±SEM). AVP (10<sup>-8</sup> M) produced sustained constriction of CPA's. Retigabine (an anticonvulsant for use in the treatment of epilepsy) and S-1 acrylamide induced dose-dependent relaxation in CPA's, compared to diluent treated controls (p<0.05, 2 way ANOVA). S-1 acrylamide induced greater relaxation of vessels pre-constricted with AVP than retigabine (p<0.05, 2 way ANOVA means±SEM; by 66±15% vs 45±14% (maximum relaxation expressed as a percentage of AVP constriction in controls), respectively. Retigabine and S-1 acrylamide relaxed arteries pre-constricted with 80mM KPSS.

Conclusion: Pre-constricted CPA's relaxed in response to two structurally distinct K<sub>V</sub>7 channel openers. S-1 acrylamide is reported to have a preferential effect on K<sub>V7</sub> channels (3) and retigabine on K<sub>V7</sub>2.5 channels [4]; therefore our data suggest that K<sub>V7</sub>4 and/or on K<sub>V7</sub>2.3.5 channels could contribute to vascular tone regulation of CPA's. In support of this proposal, we have previously localized K<sub>V7</sub>4 channel protein to the CPA endothelium and smooth muscle. Further studies will examine a possible role for altered K<sub>V7</sub>4 expression or function in the increased fetoplacental vascular resistance characteristic of fetal growth restriction.

Mills TA et al. (2009). Reproductive Sciences 16(35)163A.

Retigabine and S-1 acrylamide were a gift from NeuroSearch A/S, Ballerup, Denmark. This work was supported by Action Medical Research and the Manchester Biomedical Research Centre.

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PC240

Insulin reverses the H<sub>2</sub>O<sub>2</sub>-induced vasoconstriction in human feto-placental microcirculation

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Pregnancy is a pro-oxidative state due to increased physiological synthesis of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide radical, mainly. If placenta antioxidant mechanisms are inefficient, this organ is the source of oxidative stress, vascular dysfunction and reduced supply of nutrients and oxygen to the fetus. Insulin increases L-arginine transport via the cationic amino acid transporters (hCATs) and induces membrane hyper-polarization in human umbilical vein endothelial cells (HUVECs) [González et al. 2004]. Recently, we demonstrate that insulin induces relaxation of human umbilical vein by an hCATs-dependent mechanism (González et al., 2011). We propose that insulin reverses the placental vascular damage generated by the increase of H<sub>2</sub>O<sub>2</sub>, in a mechanism involving activity of potassium channels.

Placenta and umbilical cords were obtained from normal pregnancies (Ethics committee approval and informed patient consent were obtained). Umbilical and chorionic vessels rings were mounted on an isometric force transducer and registered the highest contractile response (90 mKCl). Once washed and stabilized, rings were pre-incubated with Krebs solution in absence or presence of insulin (10nM), wortmannin (30 μM), genistein (5 μM), L-NAME (100 μM), iberiotoxin (IBTX, 100 nM, BK channels inhibitor) and exposed (5 min) to H<sub>2</sub>O<sub>2</sub> (10<sup>-10</sup>-10<sup>-7</sup> M). Insulin relaxation assays were made in absence or presence of IBTX. Results are shown as percentage of maximal KCl-induced contraction (%KCl). For placental cotyledon perfusion assays, suitable fetal vein and artery pairs on the surface of the chorionic plate were plastic tubes cannulated. Each cotyledon was perfused with a Ringer-Krebs using a Master Flex perfusion pump (model no. 752 1-10) at constant flow rate. H<sub>2</sub>O<sub>2</sub> (1 mM, 2 min) induced significant (upaired Student’s t test, P<0.05, n=5-10) contraction of umbilical artery (27±5 %KCl), umbilical vein (41±7 %KCl) and chorionic vein (21±5 %KCl). This contraction was prevented by pre-incubation with insulin, wortmannin, genistein, and was increased by L-NAME and endothelium removal. IBTX did not alters H<sub>2</sub>O<sub>2</sub> effect. In U46619 pre-constricted veins, relaxation caused by insulin was abolished by IBTX, but not by genistein. In other hand, H<sub>2</sub>O<sub>2</sub> (10 nM) increased the perfusion pressure (3.2±0.2-fold) without changes in flow rate in placental cotyledon. Insulin (0.1 nM) induced relaxation of chorionic vessels and blocks the vasoconstriction induced by H<sub>2</sub>O<sub>2</sub>. We suggest that insulin protects the feto-placental circulation against vasoconstriction caused by acute increase in oxidative stress. Insulin effect would be related with acute relaxation of chorionic vein induced by this hormone through a mechanism involving BK channels activity without participation of tyrosine kinases.

Dynamics of ovarian follicular development and atresia are regulated by the balance of pro- and anti-angiogenic isoforms of vascular endothelial growth factor (VEGF)

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Vascular endothelial growth factor (VEGF) moderates angiogenesis and vascular permeability in the ovary. It is expressed as a pro-angiogenic (e.g. VEGF₁₆₅) or anti-angiogenic (e.g. VEGF₁₆₅b) isoform according to splice site selection in the terminal exon. Angiogenesis provides follicles with the hormonal milieu required to reach pre-ovulatory stage, whereas anti-angiogenesis accompanies atresia. VEGF₁₆₅b over-expression in the ovary(1) has been shown to reduce litter size and the number of ovulated embryos in the oviduct, but it is not known whether this was due to reduced angiogenesis in the ovaries of these mice or reduced follicle stimulating hormone (FSH) signalling.

Here we examined the number and size of healthy follicles, incidence of FSH receptor expression, number and size of CL, and the quantification of atretic follicles, in the ovaries of transgenic (TG) mice over-expressing VEGF₁₆₅b in the ovary under control of the MMTV promoter(1) and in wild type (WT) littermate controls. Animals (n = 6/group) were mated with wild type mice and killed by a schedule 1 method at 0.5 days post coitus. Using immunohistochemistry and morphometric analysis, we determined that the total number of healthy follicles was significantly reduced in TG mice compared to WT controls (4.39±1.31 vs 8.25±0.90 /section; respectively; p<0.05). TG mice had a significant decrease in the number (0.61±0.33 vs 2.25±0.38 /section; p<0.05) and size (p<0.05) of secondary-antral follicles, and a significant reduction in the ratio of tertiary to secondary-antral follicles (0.037±0.02) in comparison to WT controls (0.65±0.28; p<0.05). Furthermore, the diameter of tertiary follicles was significantly smaller in TG when compared to WT controls (p<0.05). However, TG and WT mice showed no significant difference in FSH-R expression (4.15±0.89 vs 4.02±0.34; respectively; p>0.05). TG mice were found to have a highly significant reduction in the number of CL (1.83±0.53 vs 4.70±1.09 /section; p<0.01), and a significant reduction in the size of the CL (p<0.05), compared to WT littermates. Finally, we observed a significant increase in the number of atretic follicles in the ovaries of TG mice, compared to WT controls (1.24±0.56 /section; p<0.05).

These results confirm that VEGF is a key regulator of follicular and CL angiogenesis, and show that inhibition by VEGF₁₆₅b results in reduced growth and quantity of follicles and CL. This study also provides evidence that VEGF inhibition by VEGF₁₆₅b does not affect FSH-R expression, and that VEGF regulates follicle atresia.

Insulin increases nitric oxide (NO) synthesis via the endothelial NO synthase (eNOS) and L-arginine transport via the cationic amino acid transporters (hCATs) in human umbilical vein endothelial cells (HUVECs)(González et al. 2004). Ex vivo experiments demonstrated that acute (30 min) and chronic (8 h) insulin causes relaxation of human umbilical vein by an endothelium and hCATs dependent mechanism (González et al., 2011). We here investigated the involvement of tyrosine kinases pathway in the acute effect of insulin in vascular reactivity of human chorionic vein, and chronic insulin effect on hCAT-1 expression in human umbilical vein.

Chorionic vein rings and HUVECs were isolated from normal pregnancies (Ethics committee approval and informed patient consent were obtained). Rings were mounted on an isometric force transducer and registered the highest contractile response (90 mM KCl). Vessels were washed and constricted with 100 nM U46619 (thromboxane A₂ analogue). Once stable maximum contraction was reached, rings were exposed to insulin (10 nM) in absence or presence of genistein (tyrosine kinases inhibitor) or wortmannin [phosphatidylinositol 3 kinase (PI3K) inhibitor]. Cells were isolated by collagenase digestion and cultured in medium 199 (M199) supplemented with 20% newborn and fetal calf sera. Protein abundance (western blotting) and mRNA (real time RT-PCR) for hCAT-1 were measured in absence or presence of insulin (1 nM, 8 h).

We suggest that in acute ex vivo experiments insulin induces relaxation of placental vessels in a PI3K or other tyrosine kinases independent manner. Interestingly, the relaxation induced by insulin and genistein are summative, suggesting two different

PC242

Role of tyrosine kinases activity in vasodilator mechanisms induced by insulin in human fetal vein

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PC243

Vascular Endothelial Growth Factor-A (VEGF-A) overexpression in adult mouse glomeruli increases glomerular ultrafiltration coefficient due partly to the reduced coverage of the capillaries by the subpodocyte space (SPS)

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Glomerular podocyte VEGF-A signals across the glomerular filtration barrier (GFB) to maintain permselectivity(1) and VEGF-A isoforms change in human nephropathy(2). We investigated the roles of the angiogenic mouse isoform VEGF-A164 in mature adult glomeruli in vivo using conditional inducible transgenic overexpression in mice. Procedures followed UK Home Office Legislation and had local ethics committee approval. podocin rtTA mice and tetO-VEGF-A164 mice were crossed resulting in double-transgenic (podocin rtTA x tetO-VEGF-A164: P+T+) mice. Podocyte-specific VEGF-A164 overexpression was induced in 8 to 22 week old mice by adding doxycycline to drinking water of adult P+T+ mice. Genotyping confirmed presence of the transgenes and kidney VEGF-A164 expression was measured by ELISA. The glomerular ultrafiltration coefficient (LpA) of P+T+ mice and wild type littermates (WT) was measured (3) and normalized to initial glomerular volume (LpA/V). Isolated glomeruli mounted on a pipette were bathed in 1% bovine serum albumin (BSA) in HEPES Ringer. Upon changing to 8% BSA, the efflux of water reduced glomerular volume. The initial (<0,1s) rate of glomerular volume reduction (j0) was used to calculate the glomerular ultrafiltration coefficient (LpA = j0/Δπ (nl.min-1.mmHg-1)). Small portions (<1mm diameter) of P+T+ and WT kidney were fixed with glutaraldehyde, osmicated, dehydrated and embedded in resin. Glomerular sections were examined and electron micrographs used to estimate: glomerular basement membrane (GBM) thickness, podocyte filtration slit and endothelial fenestration frequency, coverage (%) of the GBM by the SPS(4), height of SPS, width of SPS exit pores and width of subpodocyte space coverage of the glomerular capillary wall (65±4% WT; 43±6% P+T+ p<0.01). No other parameter differed between P+T+ and WT, indicating that the GFB was ultrastructurally unchanged. Using models developed by Neal et al(5), the SPS changes account for 63% of the increase in ultrafiltration coefficient. While the adult mouse GFB is relatively insensitive to induced VEGF-A164 over-expression, the podocyte appears to respond by reducing overall GFB resistance by increasing the fraction of GFB without SPS cover. This raises the ultrafiltration coefficient by allowing filtration through a greater area of the GFB that has lower resistance.

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PC244

Effects of high glucose and insulin on vasorelaxation to anandamide

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Endocannabinoids, acting via central and peripheral cannabinoid receptors, are involved in the control of food intake and energy balance. Of note, the metabolic parameters, high glucose and insulin have been shown to modulate the actions and tissue content of anandamide (an endocannabinoid) in pancreatic beta-cells, adipocytes and serum (1-2). Interestingly, anandamide is also a potent vasorelaxant and has been implicated in blood pressure regulation (3). However, the influence of glucose and insulin on the vascular effects of anandamide remains undetermined. In this study, small mesenteric artery and aorta were isolated from male Wistar rats (200-350g, killed by cervical dislocation) and maintained at 37°C in oxygenated Krebs-Henseleit solution (with 10 mM glucose) for isometric tension recording. Vessels were precontracted with 10μM methoxamine (an α1-adrenoceptor agonist), followed by cumulative additions of anandamide. Data are expressed as means±s.e.m (n=4rats) and analysed by Student’s t-tests or 2-way analysis of variance. In the aorta, high glucose (30mM for 1h) significantly reduced relaxation to anandamide (relaxation at 10μM, 10mM glucose = 40±4%; at 30mM glucose = 17±9%; P<0.05). Response to 10μM anandamide was also reduced in the presence of 0.1μM insulin (at 10mM glucose, 16±10%; P<0.05). In contrast, mesenteric relaxation to anandamide was not affected by high glucose (control: pEC50 = 6.97±0.17, Rmax = 106±9%; 30mM glucose: pEC50 = 7.03±0.19, Rmax = 106±11%).
6.78±0.17, Rmax = 104±6%). Insulin (0.1µM) also had no effect (at 10mM glucose, control: pEC50 = 6.71±0.9, 100±5%; + insulin: pEC50 = 6.70±0.15, Rmax = 109±12%). High glucose also significantly reduced the methoxamine-precontracted tone in aorta (by 39±10%), but not mesenteric artery. To conclude, high glucose and insulin reduce vasorelaxation to anandamide and vasocontraction to methoxamine, depending on the vessel subtype (conduit vs resistance arteries) or vascular region. The compromised anandamide response might contribute to vascular changes seen in hyperglycaemia and/or hyperinsulinæmia.


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**PC245**

**Functional role of Kv7.1 channels in rat mesenteric artery**

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KCNQ1-encoded voltage-dependent potassium (K+) channels (Kv7.1) underlie the slow delayed rectifier potassium current (IKs) in cardiac myocytes. Recently, these channels along with Kv7.4 and Kv7.5 have been identified in vascular smooth muscle cells (Greenwood and Ohya, 2009). This study used selective IKs inhibitors and enhancers to characterise the role of Kv7.1 channels in rat mesenteric artery. In addition, we investigated their possible contribution to β-adrenoceptor-mediated vasodilatation. Third order mesenteric arteries were isolated from male Wistar rats (200-225 g). Isometric tension recordings were performed in a wire myograph. IKs inhibitors (HMR1556, L-768,673 and JNJ39490282; Towart et al., 2009) and enhancers (L-364,373 and mafenamic acid) were used to assess Kv7.1 channel functionality. Responses to isoprenaline or forskolin were measured in arteries precontracted with 10 µM methoxamine in the presence and absence of various Kv7.1 channel blockers. Expression of KCNQ mRNA was determined using quantitative PCR. Values are presented as mean ± s.e.m., compared by two-tailed Student’s t-test. Amplicons for KCNQ1, KCNQ4 and KCNQ5 mRNA were detected readily in rat mesenteric arteries. HMR1556, L-768,673 and JNJ39490282 had no contractile effects in mesenteric arteries (under minimal tone), whereas the non-selective Kv7 blocker linopirdine caused robust contractions. In precontracted mesenteric vessels, the IKs enhancers L-364,373 and mafenamic acid caused concentration-dependent relaxation that was fully reversed by Kv7.1 blockers. Relaxation in response to isoprenaline was attenuated in the presence of the linopirdine (10 µM; pEC50, 6.5±0.3 vs. 7.2±0.1 vehicle control; n=7-10; P<0.01). Similarly, isoprenaline-induced relaxation was reversed by 10 µM HMR1556 and L-768,673. These inhibitory effects were not mimicked by the Kv channel blocker 4-aminoypyridine (1 mM) or the KATP blocker glibenclamide (10 µM). Relaxation caused by the adenyl cyclase activator forskolin was also inhibited by linopirdine (pEC50, 7.7±0.3 vs. 8.4±0.1 vehicle control; n=3-4; P<0.001).

The results from this study confirm that inhibition of Kv7.1 channels does not alter basal vascular tone. However, direct activation of Kv7.1 channels causes potent vasodilatation. Moreover, Kv7.1 channel activity appears to contribute to β-adrenoceptor-mediated relaxation in rat mesenteric artery. Greenwood IA and Ohya S (2009). Brit J Pharmacol 156, 1196–1203 Towart R et al. (2009). Journal of Pharmacological and Toxicological Methods 60, 1–10.

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**PC246**

The effects of zinc deficiency on vascular smooth muscle cell function

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Recently dietary zinc deficiency has been associated with a potential role in cardiovascular diseases and changes have been observed in the expression of proteins associated with vascular smooth muscle cell (VSMC) differentiation (Beattie et al. 2008). The aim of this study is to elucidate the effect of a zinc deficient environment on vascular smooth muscle cell function using both in vivo and in vitro models. Male Lister hooded rats (8 weeks old) were maintained on a 2 week zinc adequate diet (35 mg/kg) or zinc deficient diets (3mg/kg or <1mg/kg). There was a significant reduction in plasma zinc levels in rats fed a zinc deficient diet compared to the zinc adequate diet as assessed by atomic absorption spectrophotometry. 35mg/kg zinc diet - 150 ± 4 µg/ml, 3mg/kg zinc diet - 80 ± 4 µg/ml, 1mg/kg zinc diet - 60 ± 4 µg/ml; n=10 for each diet group, mean ± s.e.m., ANOVA p<0.05). Carotid arteries were dissected and stimulated for 15 mins with either 50 ng/ml platelet-derived growth factor (PDGF) or 1 µM sphingosine 1-phosphate (S1P), both of which have been previously shown to activate intracellular growth pathways in VSMC. Activation of extracellular signal-regulated kinase (ERK1/2) was measured by immunoblotting in arterial homogenates as an indicator of cell growth and survival. In arteries from zinc deficient rats, S1P-stimulated ERK1/2 activation was significantly decreased compared to rats fed a zinc adequate diet (densitometry; 35mg/kg zinc diet - 5.3 ± 2.0 fold increase, 3mg/kg/zinc diet - 1.4 ± 0.5 fold increase, <1mg/kg zinc diet - 1.3 ± 0.4 fold increase; n=5 for each group, mean ± s.e.m., ANOVA p<0.05). PDGF-induced activation of ERK1/2 was also reduced (35mg/kg zinc diet - 5 ± 1.4 fold increase, 3mg/kg zinc diet - 1.3 ± 0.1 fold increase, <1mg/kg zinc diet - 1.3 ± 0.3 fold increase; n=4 for each group, mean ± s.e.m., ANOVA p<0.05). The total ERK1/2 expression was unaltered in arteries from rats on zinc deficient diet compared to zinc adequate diets. To determine if these changes in ERK1/2 activation were reflected in changes to VSMC phenotype, the expression of protein markers for smooth muscle differentiation was examined. Expression of calponin and smooth muscle α-actin were unchanged in arteries from rats with zinc deficient diets. In parallel in vitro experiments, primary cultured rat aorta VSMCs were maintained for 24 hours in zinc-deficient or zinc-supplemented medium and stimulated with either PDGF or S1P for 15 mins. ERK1/2 activation was unaffected in zinc deficient compared to zinc-deficient conditions.
supplemented conditions. Also, markers of smooth muscle differentiation were unaltered. In conclusion, dietary zinc deficiency in vivo reduces activation of the growth and pro-survival pathways in arteries suggesting a change towards an apoptotic phenotype. This is probably not a direct effect of zinc deficiency on VSMC.


This work was funded by the Research Foundation of Korea (NRF).

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PC247

Calcium stimulated adenylyl cyclase modulates ion channel currents in the guinea-pig atrioventricular node

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The atrioventricular node (AVN) is essential to cardiac function, forming the only pathway between the atria and ventricles, crucially timing ventricular filling and contraction. There is currently little information available regarding ion channel regulation by intracellular second messengers, and in particular the contribution of cyclical changes in intracellular calcium to ion channel function. cAMP is fundamental to cellular function, and its levels are maintained in dynamic equilibrium via breakdown by phosphodiesterases (PDE) and production by adenylyl cyclase (AC). Calcium stimulated ACs, which have been shown to play an important role in neuronal function, have been identified in sinoatrial nodal tissue, but are absent from ventricular tissue (Mattick et al., 2007).

Adult male guinea-pigs (~400g) were killed by cervical dislocation. The heart was rapidly removed and retrogradely perfused with a collagenase solution using a modified langendorff apparatus. The AVN region was excised and cells isolated using previously described methods (Hancox et al., 1993; Yuill et al., 2010). Here we present evidence of calcium dependent AC activity in individual atrioventricular nodal myocytes from the guinea-pig, using perforated patch clamp electrophysiology to record ionic currents and action potentials, and confocal line scan imaging with Fluo-5 to record spontaneous calcium transients. Experiments were only conducted on healthy AVN myocytes exhibiting regular spontaneous activity. All recordings were carried out at 37°C, using standard physiological potassium-based solutions. Statistical analysis was performed using a paired t-test, with Bonferroni post-hoc correction.

Inhibition of sarcoplasmic reticulum activity with cyclopiazonic acid (10μM) or ryanodine (1μM) induced a cessation in action potential firing, highlighting the importance of spontaneous calcium release to the generation of spontaneous electrical activity in AVN myocytes. We demonstrated the functional importance of calcium dependent AC activity on cellular electrical activity and calcium release, by addition of inhibitors of AC (MDL 12330A) and PDE (IBMX) activity. Application of 10μM MDL 12330A significantly attenuated spontaneous action potential firing rate, induced a negative shift in the activation of a hyperpolarisation activated non-selective cation current ('funny current', If), inhibited L type calcium channel (ICaL) amplitude (65.3 ± 2.3%, S.E.M, n=5), and reduced the frequency of spontaneous calcium release (P=0.05, n>5).

Conversely, IBMX caused a positive shift in the voltage dependence of If activation and an increase in the frequency of spontaneous calcium release. These effects are consistent with the presence of calcium dependent AC in AVN myocytes, which is integral to the generation of spontaneous electrical activity in these cells.


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PC248

Elevated extracellular glucose markedly attenuates ischaemic preconditioning in cardiac ventricular myocytes

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Elevation of blood glucose is associated with worsening of outcome for patients after acute myocardial infarction(1). Hyperglycaemia has been implicated as causative in a number of deleterious vascular complications and has recently been shown by us, and others, to affect cardiac tissue; in particular being pro-arrhythmic and to attenuate the protective effect of prodromal angina. In this study we have investigated the effects of elevations of extracellular glucose on the protection to cardiomyocytes afforded by ischaemic preconditioning. Ventricular myocytes were enzymatically isolated from excised hearts from adult male Wistar rats (animals were sacrificed in accordance with Home Office regulations). Excised hearts were ischaemically preconditioned (IPC) on a Langendorff system by interrupting perfusion 3 times for 5 minutes with 5 minutes of reperfusion between each stop(2). Cells were then isolated after the 3rd reperfusion.

Cardioprotection was assessed by contractile recovery after a metabolic inhibition/reperfusion protocol mimicking ischaemia and reperfusion (I/R). Briefly, cells were perfused with 5 mM glucose containing normal Tyrode solution (NT) at 32±2°C and paced to contract at 1 Hz with electric field stimulation. This was followed by 7 minutes of substrate-free metabolic inhibition Tyrode (SFT) containing 2 mM cyanide and 1 mM iodoacetic acid. Finally the cells were reperfused with NT and contractile recovery was assessed at 10 minutes. In control cells 32±4% (n=112) of cells recovered their contractile function which increased to 88±3% (n=93) after IPC (P<0.001). Increasing extracellular glucose concentration markedly reduced the protection afforded by IPC, (10mM, 76±5% (n=74), 15mM, 70±4% (n=118) and 20mM, 56±5% (n=89)). In 20mM glucose 59±5% of control cells (i.e. those not subjected to IPC) recovered their contractile function suggesting that high glucose had abolished the additional protection afforded by IPC. These data suggest that hyperglycaemia abolishes certain mechanisms of cardioprotection in ventricular myocytes and that this is inversely proportional to the extracellular glucose concentration between 5-20mM. Previous data from our laboratory demonstrate that phorbol ester induced PKC-mediated cardioprotection (3) is markedly attenuated by elevated extracellular glucose. Such attenuation of cardioprotection is of clinical importance in defining the regulation of glycaemia in patients presenting with myocardial infarction. Increased understanding of the mechanisms by which cardioprotection

188P
Effects of Physiological Parameter Variation in a Computational Rabbit Ventricular Cell Model

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INTRODUCTION: Computational cardiomyocyte models are provided with a single, deterministic parameter set. This parameter set is composed of variables that are determined either directly by experimentation, or based on phenomenological fitting of several parameters at once, comparing the output of the model to experimental data. Ion current conductances are especially subject to this, as experimental methods are unable to measure them directly, forcing indirect measurement using drug block, which in itself can produce artefacts in the data. Consequently, the parameter set of a model may not reflect physiological reality. Furthermore, a deterministic parameter set does little to address physiological variation between individuals. Thus, we investigated the effect of variation in ion channel peak conductance in a state-of-the-art rabbit ventricular cell model by conducting a simultaneous sweep of peak conductance values ranging from -30 to +30% for six ion channel types. The current study can be described as a state-of-the-art rabbit ventricular cell model by conducting a simultaneous sweep of peak conductance values ranging from -30 to +30% for six ion channel types. The resulting conductance parameter variation may be a source of physiological variation between individuals. Furthermore, the response of ion channel conductance to drug block may be counter-intuitive, as a result of non-linear interactions between various intracellular ion concentrations and ion channel populations.

PC249

The Energetics of Cardiac Trabeculae Undergoing Quasi-Realistic Work-Loops

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An unresolved issue in the field of cardiac energetics is the want of explanation for the well-documented linear relationship between cardiac energy expenditure and pressure-volume area (PVA). PVA is given by the sum of the pressure-volume-time ‘work loop’ and the triangular region lying to its left between the end-systolic and end-diastolic pressure-volume relations. In order to address this issue, we have developed a unique flow-through micro-mechano-calorimeter that is capable of measuring, simultaneously, both the force and the heat produced by actively contracting ventricular trabeculae3,4. Adult rats were deeply anaesthetised with isoflurane and their hearts removed. A geometrically-uniform, free-running, right-ventricular trabecula was dissected and mounted in the calorimeter. Mechanical and thermal measurements were made at room temperature in response to various stimulus frequencies. The superfusate was a modified Krebs-Henseleit solution containing 1.5 mM [Ca2+]o. Trabeculae underwent both isometric and fixed-end contractions, as well as quasi-realistic (‘rectangular’) force-length loops designed to mimic pressure-volume loops generated by the heart in vivo. We found the force-length relationship of trabeculae to be non-linear in response to both fixed-end and isometric contractions. The same non-linearity prevailed when preparations underwent contact for preload or variable after-load. Despite this non-linearity, the heat versus force-length area (FLA) relation was linear, in accord with VO2-FLA results from ferret papillary muscles5 and VO2-PVA results from canine whole-hearts6. There can be little doubt that the phenomenon is a characteristic of cardiac myocytes per se and is not an emergent property of the three-dimensional whole-heart. We are currently developing mathematical models with the aim of understanding its cellular origin.


PC251

**Computer Simulation of Human Atrial Fibrillation due to S140G and V141M Mutations of the K\textsubscript{v}7.1 Gene**

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Mutations of the slowly activating delayed potassium ion current (I\textsubscript{Ks}) regulating gene K\textsubscript{v}LQT1 (K\textsubscript{v}7.1) have been implicated in the genesis of atrial fibrillation (AF). This computational study quantifies the mechanisms by which altered I\textsubscript{Ks} ion current function affects cell and tissue level electrical behaviour. Control and mutant atrial I\textsubscript{Ks} voltage clamp experimental data [1] were analysed to quantify alterations of maximum conductance (g\textsubscript{Ks}) and steady state activation parameters of voltage half activation, (V\textsubscript{1/2}) and slope (k) (Fig. 1, A and B). The time kinetics were found to be unaltered in the Hodgkin Huxley formulation of I\textsubscript{Ks} The estimated parameters were incorporated into the Courtemanche et al. [2] (CRN) cell model to simulate alterations of action potential (AP) profiles and underlying ionic currents due to the mutations. The cell models were further incorporated into 1D strands and 2D sheets of homogeneous atrial tissue models to study the effects of the mutations on tissue level conduction propagation behaviour [3].

Under Control conditions, g\textsubscript{Ks} is 0.129 nS/pF, V\textsubscript{1/2} is 19.1 mV, and k is 12.7 mV. The S140G experimental data gave g\textsubscript{Ks} to be 0.04128 nS/pF, V\textsubscript{1/2} to be -29.94 mV, and k to be 14.9 mV. The V141M gave a g\textsubscript{Ks} of 0.0258 nS/pF, V\textsubscript{1/2} of -21.13 mV, and k of 22.75 mV. Upon incorporating the Control and mutant I\textsubscript{Ks} ion current models into the CRN AP model, AP duration (APD) was reduced from 312.14 ms under Control conditions, to 232.17 ms under S140G conditions, and 255.08 ms under V141M conditions (Fig. 1, C). Both mutations increased the maximum slope of APD restitution. Cellular effective refractory period (ERP) was reduced from 380 ms in Control, to 300 ms under S140G conditions, and 323 ms under V141M conditions. In 1D models, solitary wave CV was found to be 0.27 mm/ms under Control conditions, and reduced to 0.255 mm/ms (S140G) and 0.265 mm/ms (V141M) under mutant conditions. CV restitution revealed that the tissues’ capacity to sustain high pacing rate conduction waves increased from 350 ms (Control) to 179.8 ms (S140G) and 246.2 ms (V141M) (Fig. 1, D). In 2D simulations, the re-entrant waves self terminated within 1.8 ms while under S140G and V141M conditions the re-entry persisted for the duration of simulated 10 s. The re-entrant wave tips were stable under the mutant conditions. The mutations reduced cellular APD and ERP dramatically. The mutations reduced CV while the tissues’ ability to sustain conduction propagation at high pacing rates was increased. The reduced APD and CV gave rise to a reduced wavelength of propagating waves, indicating the augmented propensity of tissue level AF due to the gene mutations. This is reflected in the 2D simulations where stability of re-entry was drastically increased.

Figure 1. Experimental data and model simulations. In all panels, filled circles represent experimental data under Control conditions, gray squares represent V141M experimental data, and triangles denote S140G experimental data. Solid lines show model simulations for Control, gray lines for V141M, and dashed lines for S140G. A: Steady state of I\textsubscript{Ks} activation. B: I\textsubscript{V} relationships under Control and mutant conditions. C: Simulated AP profiles. D: CV restitution.


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PC252

**Mechanical and electrical effects of superoxide donor menadione in rat myocardium are associated with increased diastolic intracellular Ca\textsuperscript{2+}**

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Reactive oxygen species such as superoxide are involved in many cardiac diseases. Menadione is a superoxide donor that has varied effects upon the mechanical and electrical activity of cardiac muscle(Anderson & Dutta 1991; Choi et al, 2005). It has been suggested that its effects are associated with changes in intracellular Ca\textsuperscript{2+} but this has not been tested nor have simultaneous mechanical and electrical responses been recorded. The aim of this study was to test the hypothesis that the mechanical and electrical effects of menadione occur in the presence of dysfunctional intracellular Ca\textsuperscript{2+} regulation.

Male Wistar rats 200-250g were killed, hearts removed and Langendorff perfused with a Krebs-Heinsliebt solution at 37 °C. Left ventricular pressure was monitored by an indwelling balloon connected to a pressure transducer and monophasic action potentials (MAPs) were recorded from the epicardial surface of the left ventricle. Hearts were alternately paced 5Hz or allowed to intrinsically pace for 5 min periods before and during a 30 min exposure to 50 μM menadione (dissolved

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in 0.01% vol/vol methanol). Single left ventricular myocytes were loaded with the fluorescent Ca2+ indicator fura-4 AM and intracellular Ca2+ transients and cell length simultaneously measured in cells stimulated at 1Hz before and during exposure to menadione. Data are presented as means ± SEM.

In paced preparations exposure to menadione caused a significant increase in diastolic pressure of 50 ± 9.9 mm Hg (P < 0.001); decrease in developed pressure of 59 ± 14.4 mm Hg (P < 0.001) and rate of relaxation by 886 ± 241 mm Hg s−1 (P < 0.001). There was also a significant reduction in MAP duration at 80% repolarisation by 48 ± 7.8% from an initial mean duration of 26.4 ± 2.3 ms (P < 0.001) (1-way RM ANOVA). When hearts were not paced, intrinsic heart rate fell significantly following exposure to menadione by 82 ± 21 beats min−1 (P < 0.05, Student’s paired t-test) and heart rate became less regular, as indexed by its S.D. (P < 0.05, Wilcoxon signed rank test) (N = 7 hearts). These effects did not reverse upon wash and were not observed in hearts exposed to vehicle alone (P > 0.05, N = 4 hearts). In single myocytes, menadione caused a significant increase in diastolic Ca2+ (P < 0.05) and a decrease in resting cell length (P < 0.001) (n = 5 cells, N = 3 animals, Student’s paired t-test).

Our observations are consistent with menadione causing dysregulation of Ca2+ handling possibly via effects on the sarcoplasmic reticulum Ca2+-ATPase (SERCA2) and intracellular Ca2+ overload which leads to an increase in diastolic Ca2+ and diastolic contracture together with a shortening of the action potential, possibly via Ca2+-induced inactivation of L-type Ca2+ channels.


ALLR and KW contributed equally to this work.
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PC253

Effects of anandamide on electrical activity and contraction in guinea pig cardiac ventricular myocytes
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The endocannabinoid, anandamide, not only reduces left ventricular developed pressure in isolated hearts (Ford et al., 2002), but also has anti-arrhythmic effects (Krylatov et al., 2002). Although the actions of endocannabinoids are generally attributed to their association with cannabinoid CB1 or CB2 receptors, the identity of the receptor(s) or target(s) mediating the cardiac effects of anandamide are unknown. Objectives of this study were to investigate the effects of anandamide on electrical and contractile activity of isolated guinea pig ventricular myocytes, and to characterise pharmacologically any receptor(s) involved.

Myocytes were stimulated to fire action potentials by a 2 ms depolarising current pulse applied via an intracellular micro-electrode at a frequency of 1 Hz, and contraction measured using an edge detection system. Whole cell L-type Ca2+ currents were measured using the single electrode voltage clamp technique. Data are means ± S.E.M., compared by ANOVA.

Exposure of myocytes to anandamide (1, 3, 10 μM) resulted in a concentration dependent reduction in action potential duration (APD). After 10 minutes, 10 μM anandamide reduced APD at 90 % repolarisation by 39 ± 7 % (n = 7, p < 0.001), which was accompanied by a reduction in the amplitude of myocyte contraction. Furthermore, the peak amplitude of L-type Ca2+ currents was reduced by 57 ± 11 % at 0 mV (n = 6, p < 0.01). In a separate set of experiments, 10 μM R(+)-methanandamide (a non-hydrolysable analogue of anandamide) caused a similar reduction in APD (34 ± 6 % at 90 % repolarisation; n = 6, p < 0.001) and amplitude of contraction, showing that the actions of anandamide appear not to occur as a result of hydrolysis to arachidonic acid. The reduction in APD in response to anandamide (10 μM) was partially inhibited by the CB1 receptor antagonist AM 630 (1 μM), but the effects were smaller than predicted based on a K i value of 31 nM for CB1 receptors. The CB1 receptor antagonist AM 281 (1 μM), the vanilloid VR1 receptor capsazepine (1 μM) and O-1918 (1 μM; an antagonist of the proposed ‘non CB1/non CB2’ receptor; Offertáler et al., 2003) failed to inhibit the response. In addition, the selective CB1 receptor agonist ACEA (10 μM) and the selective CB2 receptor agonist HU-308 (10 μM) were without significant effect on APD compared to equivalent concentrations of vehicle.

Based on these data, the reduction in APD and contraction observed can, at least in part, be accounted for by the inhibition of L-type Ca2+ currents in response to anandamide. However, additional effects on other ion channels or intracellular targets cannot be ruled out. It appears that if a cannabinoid receptor mediates these effects, the characteristics of this receptor are not those expected of conventional CB1 or CB2 receptors.

Ford WR et al., (2002). Br J Pharmacol 135, 1191-1198

This work was supported by the British Heart Foundation.
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PC254

The effect of inorganic phosphate on mitochondrial Ca2+ dynamics in single permeabilized ventricular myocytes of rat
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Mitochondrial dysfunction affects cell viability because mitochondria are related to both necrosis and apoptosis following ischemia and reperfusion. Loss of ATP and increased ATP hydrolysis, impaired ionic homeostasis are recognized as key factors of mitochondrial damage in the generation of cell death. Mitochondria can absorb huge amount of Ca2+ by buffering action of inorganic phosphate (Pi). In this study, we would like to see the effect of inorganic phosphate on mitochondrial Ca2+ dynamics with newly developed quantitative method. We enzymatically isolated single ventricular myocytes of rat. All procedures were accorded with national legislation. Buthane dione monoxime was present to prevent contracture. We measured NADH, mitochondrial Ca2+ with Fura-2ZFF and mitochondrial membrane potential (ψm) with tetramethylrhodamine ethyl ester simultaneously. The application of 0.5 μM Ca2+ partially depolarized ψm and increased the mitochondrial matrix Ca2+. The application of 20 mM Pi only hyperpolarized ψm, and the addition of Ca2+ almost completely depolarized ψm rapidly.
And also mitochondrial matrix Ca$^{2+}$ loading was substantially decreased. The K+ substitution with N-methyl-D-glucamine or the application of tetraethyl ammonium partially slowed depolarization speed and these results suggests the depolarization may be caused by K+ dependent manner. The depolarization was not blocked by cyclosporine or 5-hydroxy decanoate, which implied that this was not related to mitochondrial permeability transition pore or mitochondrial ATP sensitive K+ channel activation. One of possible causes is Ca$^{2+}$-dependent K+ channel activation, however, it cannot explain all the effects. The adenylic nucleotides, ATP or ADP could protect this depolarization. In conclusions, matrix Ca$^{2+}$ and Pi somehow cause the depolarization and this effect may be caused by the formation of calcium phosphate complex or cyclosporine-independent non specific pore opening. Pi plays an important role on mitochondrial Ca$^{2+}$ dynamics.

work was supported by the grant from MWHF (No. A084776)

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PC255

Is SERCA overexpression anti- or pro-arrhythmic?

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Sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) gene therapy improves mechanical function in heart failure and is under evaluation in a clinical trial. Recent work has shown that SERCA2a gene delivery to rats in a chronic heart failure model reduced both spontaneous and catecholamine-induced arrhythmias in vivo, as well as reducing sarcoplasmic reticulum (SR) Ca$^{2+}$ leak in failing myocytes. For the overexpression of SERCA, rats (male, 200–250g) received a 300μL tail vein injection of AAV9.SERCA2a (2x10¹¹ drp) into their right internal jugular vein (performed under general anaesthesia with 2% isoflurane). Six to eight weeks later, hearts from control (n = 6) and normal+SERCA (n = 5) were harvested and myocytes were enzymatically isolated from the left ventricles. Intracellular Ca$^{2+}$ transients and SR Ca$^{2+}$ leak were measured using Fluo-4 fluorescence (and expressed in terms of $F/F_0$) and cellular contraction was detected using an edge tracking system. Values are mean ± S.E.M., compared with either Student’s T-test or ANOVA with Tukey’s post-test comparison as appropriate.

The overexpression of SERCA did not cause any significant changes in electrically stimulated contractions or Ca$^{2+}$ transients (amplitude, rise time, 50 % decay). There was an increase in the SR load measured as peak fluorescence following 20 mM caffeine application (from 9 ± 0.6 in control myocytes, n = 30, to 13.2 ± 0.9 in normal+SERCA myocytes, n = 11, P < 0.01). In addition, there was a reduction in the tetracaine dependent SR leak (from 3.0 ± 0.2 in control myocytes, n = 30, to 2.3 ± 0.2 in normal+SERCA myocytes, n = 11, P < 0.05).

In control myocytes, 30 nM isoprenaline increased cell shortening by 129 ± 24 % (n = 6, P < 0.001) and Ca$^{2+}$ transient amplitude by 20.1 ± 3.3 % (n = 11, P < 0.001). In normal+SERCA cells, 30 nM isoprenaline increased cell shortening by 177 ± 45 % (n = 6, P < 0.001) and Ca$^{2+}$ transient amplitude by 26.3 ± 9.5 % (n = 6, P < 0.05). However, there was a significant increase in the diastolic Ca$^{2+}$ in normal+SERCA cells (25.4 ± 6.1 %, n = 11, P < 0.05), which means that the increase in Ca$^{2+}$ transient amplitude is underestimated by the $F/F_0$ value.

The increase in diastolic Ca$^{2+}$ in normal+SERCA cells is due to a large increase in SR leak in the presence of isoprenaline (from 2.3 ± 0.2 to 4.1 ± 0.6, n = 11 for both, P < 0.05). In addition to the increased leak, there was a greater frequency of Ca$^{2+}$ waves following isoprenaline in normal+SERCA myocytes (from 0.018 ± 0.018 to 0.303 ± 0.054 waves per second, n = 11 for both, P < 0.001). There was no increase in either SR leak or Ca$^{2+}$ wave frequency in control myocytes treated with isoprenaline. In conclusion, SERCA overexpression can be both pro- and anti-arrhythmic, depending on the type of myocyte that it is administered to (healthy vs. failing) as well as on the inotropic state of the myocyte.

We would like to thank the BHF, Leducq foundation and Wellcome Trust for their generous support.

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PC256

STED microscopy reveals secondary RyR cluster morphology

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Conventional methods used to examine antibody labelled proteins are impeded by the resolution limit of light microscopy. We report new measurements of ryanodine receptor (RyR) clusters using the superior spatial resolution of stimulated emission depletion (STED) microscopy. Cardiac myocytes from sheep atrial cells were fixed using paraformaldehyde (2%) and labelled with a primary antibody for the RyR followed by a fluorescent secondary Atto 647N. Two pulsed lasers were used for STED microscopy; a 635 nm (Coherent), pulsed at 80 MHz for 80ps was used to excite and a 780 nm laser, (Mai Tai-Deep See, Spectra physics), with a donut shaped beam was pulsed at 80MHz for 100fs for depletion. This allowed a ~ 3.5 x increase in resolution. After deconvolution, further improvements in both signal-to-noise ratio and resolution were observed. Fitting of the smallest detectable clusters showed a >4x improvement in resolution, allowing a typical lateral resolution of 40-65 nm. Using the protein size predicted from ultrastructure and as documented in rat ventricular myocytes, calculations of cluster size were possible, yielding a mean cluster size of 21 RyRs, with a standard deviation of 33. Over 50% of the clusters measured had a size of 6 or less, but many small clusters were grouped in a secondary level of organisation, resembling that typically observed after deconvolution of conventional confocal images. Doublets of RyR clusters were observed on the outer membrane of the atrial cells in agreement with previous reports, but a more complex primary structure was shown to underlie these. This method allows far-field measurement of RyR cluster morphology with near single protein resolution. Gaining new information on the geometry of peripheral and dyadic cluster geometry are now possible and may inform future avenues of study.

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Can stimulation frequency modulate Ca\textsuperscript{2+} handling of sheep atrial myocytes?

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The human atrium is the site of the most common cardiac arrhythmia observed in clinical practice, atrial fibrillation (AF). It is hypothesised that abnormal Calcium ([Ca\textsuperscript{2+}]) handling in the atrial myocardium can lead to the pathogenesis of AF. At present no data exists on the effect of pathological stimulation frequencies on atrial Ca\textsuperscript{2+} handling. We sought to investigate Ca\textsuperscript{2+} handling in young adult sheep atrial myocytes, across a range of stimulation frequencies. Atrial myocytes were isolated from the left auricle of healthy young adult sheep. Experimental protocols were carried out under perforated patch, voltage clamp conditions at 37°C. Intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) was measured using Fluo-5F at stimulation frequencies of 1, 3, and 5Hz. An increase in the rate of stimulation resulted in a significant decrease in Ca\textsuperscript{2+} transient amplitude (264 ± 74 nmol/L, 194 ± 82 nmol/L, 106 ± 51 nmol/L at 1, 3, and 5Hz respectively; p<0.005). This decrease in Ca\textsuperscript{2+} transient amplitude was accompanied by a rise in diastolic Ca\textsuperscript{2+} levels over the same frequency range (115 ± 32 nmol/L, 154 ± 27 nmol/L, 198 ± 27 nmol/L at 1, 3, and 5Hz respectively; p<0.001). To elucidate the mechanisms responsible for the observed decrease in Ca\textsuperscript{2+} transient amplitude, we examined the two main determinants of this, sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content, and L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}). SR Ca\textsuperscript{2+} content demonstrated a trend to increase with stimulation frequency (61.7 ± 10.8 μmol/L\textsuperscript{-1}, 84.9 ± 20.9 μmol/L\textsuperscript{-1}, 100.0 ± 37.4 μmol/L\textsuperscript{-1}, at 1, 3, and 5Hz respectively; p=0.054). A decrease in I\textsubscript{Ca,L} was observed at 5Hz in comparison to 1Hz (0.58 ± 0.22 vs. 1.33 ± 0.35 pA/pF, respectively; p<0.005). Similar qualitative results for Ca\textsuperscript{2+} transient amplitude, I\textsubscript{Ca,L}, and SR Ca\textsuperscript{2+} content were observed in old sheep. At pathological rates that mimic those observed in human AF, significant changes in Ca\textsuperscript{2+} handling during cardiac excitation contraction coupling (ECC) of young adult sheep are observed. These changes may be the cause of the arrhythmogenesis that is observed in human AF. Further investigation under current clamp conditions is required to determine if similar alterations in atrial Ca\textsuperscript{2+} handling are observed.

supported by Biotechnology and Biological Sciences Research Council (BBSRC).

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Impact of in-utero and postnatal exposure to a high fat nutritional environment on clock and clock-controlled genes in murine hearts

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The prevalence of the metabolic syndrome, which represents a cluster of cardio-metabolic risk factors, is increasing at an alarming rate. It has previously been shown that suboptimal in-utero and postnatal nutritional environments can increase the offspring’s susceptibility to the metabolic syndrome in adulthood (1-3). Emerging evidence demonstrates the role of the circadian clock system in the pathogenesis of the metabolic syndrome (3). In this study, we examined whether in-utero and postnatal exposure to high fat nutritional environment can alter the expression pattern of clock and clock-controlled genes in the adult offspring heart. Female C57/BL6J mice were fed either a high fat (HF, 45% kcal fat) or control (C, 21% kcal fat) diet pre-conception and throughout pregnancy and lactation. Weaned offspring were fed the HF or C diet, generating the dam-offspring dietary groups: C/C, C/HF, HF/C, HF/HF. Whole hearts were taken from 15-week old male offspring killed at 6 time points over a 24h light-dark period (n=5-6 per time point per treatment group). Initially, we examined the effect of our experimental treatments on the stability in expression of housekeeping genes (HKGs) in the offspring heart, to determine those suitable for use as reference when analysing genes of interest. We then determined gene transcript levels for the clock genes, CLOCK and PER2, and clock-controlled genes, PAI-1 and SIRT1, using quantitative real-time PCR. The stability in expression of the HKGs (analysed using the geNorm qBasePLUS software) in the adult offspring heart was affected by pre- and post-natal exposure to HF, as well as the time of sampling over the 24h period. We found that the most stable HKG were β-actin and YWHAZ. Expression levels for CLOCK was found to be 1.5 (p<0.001) and 1.4 fold higher in HF/C and HF/HF groups, respectively, vs. C/C (analysed by ANOVA). PAI-1 levels were 2.3 fold higher (p<0.001) in HF/HF vs. C/C, and for PER2 this was 1.6 fold higher (p<0.05) in HF/C vs. C/C. No differences in expression levels were observed for SIRT1 between treatment groups. Cosinor analysis showed that pre- and post-natal exposure to HF diet resulted in phase shifting in peak expression of CLOCK, PER2 and PAI-1 genes. SIRT1 also showed a phase shift in peak expression but only in the C/HF group, suggesting that prenatal HF exposure may prevent the phase shift brought about by post-weaning HF feeding. The results suggest that rhythmic expression of clock and clock-controlled genes can be disrupted following early life exposure to maternal HF nutrition, and could be further modified by post-natal HF feeding. These changes may have deleterious effects on cardiovascular function, increasing cardiovascular risk associated with the metabolic syndrome in adulthood.

Bruce et al. (2009) Hepatology 50:1796-808
Telethonin regulates transverse (t)-tubule structure and Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) in mouse ventricular cardiomyocytes

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CICR is critical for contraction in cardiomyocytes. The t-tubule system promotes the proximity of the L-type Ca$^{2+}$ channels (LTCC) and the Ryanodine receptors. T-tubule disruption is implicated early in the pathogenesis of heart failure (1). It is known that telethonin (T-Cap) is necessary for normal t-tubule structure in skeletal muscle, and that it can promote t-tubule formation in response to increases in load (2). We hypothesised that T-cap was important for normal t-tubule structure and CICR in ventricular cardiomyocytes. We studied Ca$^{2+}$ handling and t-tubule structure in ventricular cardiomyocytes from hearts lacking T-Cap (KO) at a young (<3 months) and advanced (>8 months) age compared to age-matched wild-type mice (WT). We used non-parametric t-tests. Using confocal microscopy in single cells stained with Fluo-4, we observed an increase in the variance of the time to peak of the Ca$^{2+}$ transient (P=0.005 and old KO mice (WT 308±156 ms (n=48) vs. KO 430±165 ms (n=43), p<0.05) suggesting a deterioration in the regulation of the Ca$^{2+}$ transient. In old animals, there was a delayed time to peak of the Ca$^{2+}$ transient (WT 35±20 ms (n=48) vs KO 95±18 ms (n=43), p<0.01) as well as impaired time to 90% decline (WT 375±73 ms (n=43) vs. KO 460±55 ms (n=20), p<0.05). To probe for local CICR, we recorded Ca$^{2+}$ sparks and observed an increase in Ca$^{2+}$ spark frequency in young (WT 0.346±0.35 sp/s (n=32) vs KO 0.867±0.5 sp/s (n=29), p<0.05) and old KO mice (WT 0.370±0.3 sp/s (n=15) VS. KO 0.843±0.8 sp/s (n=23) p<0.001). There was also a significantly reduced Ca$^{2+}$ spark peak in older KO animals (WT 1.90±0.3 F/F0 (n=45) vs KO 1.4±0.8 F/F0 (n=43), p<0.05). In addition, we studied LTCC density using whole cell patch clamping and observed a significant reduction in peak LTCC current density in old KO mice (WT -4.75±0.38 pA/pF (n=16) vs KO -3.37±0.30 pA/pF (n=20), p<0.001). The action potential morphology was unchanged. To examine changes to the t-tubule structure may be involved in the impaired Ca$^{2+}$ handling and reduced LTCC observed in cardiomyocytes lacking T-Cap, we used di-8-Anepps and confocal microscopy. We observed a reduced t-tubule density in old KO animals (36.8±5% (n=45) vs. 30.2±4% (n=44), p<0.05). We measured t-tubule regularity by comparing the peak of the power-frequency Fourier transform in di-8-Anepps images; we observed a less regular t-tubule structure in both young (WT 2.350±1.09 (n=30) vs KO 1.570±1.06 (n=32) and old (WT 2.10±1.10 (n=39) vs KO 1.30±1.10 (n=20), p<0.05) KO animals. T-tubule disarray may explain the changes to Ca$^{2+}$ homeostasis observed, but the demonstration of a causal link requires further studies. T-Cap is essential for normal t-tubule structure and CICR in ventricular cardiomyocytes.
The neurodynamic characteristics investigated in the present study did not significantly correlate with age in males or females. In addition, analysis of psychophysiological and neurodynamic characteristics did not reveal any significant relationships with HRV indices in either position. In conclusion, the data presented here supports the hypothesis that the heart rate decrease associated with long term vigorous exercise is only partially due to vagal withdrawal, and thus another mechanism may play a role.

Figure 1. Correlation between age and heart rate variability characteristics during voluntary and paced breathing (straight line non-linear regression). SVB - supine voluntary breathing; STVB - standing voluntary breathing; SPB - supine paced breathing; STPB - standing paced breathing.

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**PC261**

**Relationship Between Seismocardiography and Echocardiography for Measuring Cardiac Cycle Timing Events**

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Seismocardiography (SCG) has been studied for over 60 yr but has been impractical until recently because of advances in computer software and micro-processors. SCG records the low frequency vibrations induced by the heart, and records cardiac cycle mechanics and timing events. Echocardiography (Echo) is recognized as a standard for measuring cardiac performance but can be technically difficult and expensive. Limited data is available that has compared these techniques (1,2). Thus, the purpose of this study was to compare SCG with Echo to determine whether SCG could provide a valid measure of cardiac performance. We hypothesized that SCG would correlate with Echo cardiac timing events. Simultaneous SCG and Echo was performed on 28 (17 females) reportedly healthy participants (Mean ± SD; age=39.3±13.9 yrs; age range = 12-59 yrs). The SCG was recorded under resting conditions, with participants in the supine position, with a tri-axial accelerometer attached to the skin using solid gel electrodes placed over the sternum of the chest. Following SCG collection, standard Doppler and M-mode Echo measurements were collected with resulting images confirmed by an echocardiologist. Data analysis was performed on 10 cardiac cycle SCG’s and 1-3 Echo images per timing variable. The results showed the % differences between the Echo and SCG for all comparable variables ranged from 0.90% to 11%, with the coefficient of variation similar between methods. Independent T-tests (p<0.05) showed no statistically significant differences between the Echo and SCG, respectively, for aortic valve open to acceleration time (rapid ejection) of systole (71 ± 13 vs. 76 ± 12 msec), mitral valve open to E-wave (111 ± 33 vs. 110 ± 20 msec), E-wave to A-wave (370 ± 118 vs. 410 ± 144 msec), mitral valve open to E-wave (159 ± 20 vs. 164 ± 28 msec), and mitral valve open to mitral valve close (536 ± 129 vs. 573 ± 148 msec). Statistically significant differences were found for aortic valve open to aortic valve close (292 ± 18 vs. 264 ± 20 msec), and isovolumic relaxation time (81 ± 15 vs. 91 ± 8 msec). However, the Bland-Altman plot for aortic valve open demonstrated an absolute difference between the methods of 13.4 msec. These data suggest that SCG reliably measured cardiac performance when compared to Echo timing, suggesting that SCG could be used in applied and clinical research trials. Furthermore, the amplitude of the specific cardiac waveforms appeared to reflect the contractile nature of the heart.

Wilson et al. 1993, Am J Cardiol, 71:536-545
Crow et al. 1994, Am J Noninvas Cardiol, 8:39-46

We wish to thank the volunteer subjects for their participation in this study. Financial support was provide, in part, by a grant from the Canadian Institutes for Health Research, the Regina Qu’Appelle Health Region, and Heart Force Medical which also provided the dBG-300 ballistocardiograph. We also wish to thanks Dr. E. Busse for this assistance with this research.

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**PC262**

**Location of the tetraalkyl ammonium (TAA) blocking site in the mouse cardiac ryanodine receptor (mRyR2)**

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In the absence of structural details for the RyR2 pore we have developed an analogy model using KcsA as a template (1). Here we test the model’s prediction that the cytosolic cavity of RyR2 is lined by hydrophobic residues of the 10th transmembrane domain (TM10) by monitoring block by large TAA’s. We hypothesise that increases in hydrophobicity of TAA’s will result in higher affinity block due to decreased rates of dissociation (koff). An ensuing prediction is that TM10 mutants with decreased hydrophobicity (I4867A, F4870A and L4873A) will have reduced affinities for TAAs owing to increases in koff. mmRyR2 was expressed and purified as described previously (2) and incorporated into phosphatidylethanolamine bilayers for voltage clamp experiments. Single channel recordings were made in symmetrical 610 mM KCl, 20 mM HEPES, 10 μM Ca²⁺ at pH 7.2 and 22°C. To characterise block, channels were activated to open probabilities.
the RyR2 pore. hydrophobic residues of TM10 lining the cytosolic cavity of that blockade by TAAs is stabilised by interactions with 93.5 ± 52.3 s−1, p<0.05, n=5-8). Our data support the proposal ± 5.6 ± 1.8; F4870A: 62.4 ± 9.1 and L4873A: 93.5 ± 5.6 ± 1 s−1, p<0.05, n=5-8). Single channel K+ conductance was not affected by substitution of TM10 hydrophobic residues with alanine however the ability of EMD to fully activate the channel was compromised in some cases. The influence of lowering residue hydrophobicity was characterised by monitoring block by TPeA in EMD-activated and/or ryanodine-modified channels. In channels that could be activated by EMD residue substitution resulted in a lowering of TPeA affinity as the result of a significant increase in koff (WT: 18.5 ± 1.1; I4861A: 26.4 ± 2.0; I4862A: 45.6 ± 5.0; L4865A: 21.2 ± 0.9 ± 1 s−1, p<0.05, n =5-6). Following ryanodine modification all mutants exhibited a decreased affinity for TPeA and the majority exhibited significant increases in koff (WT: 35.6 ± 0.9; I4861A: 77.4 ± 6.1; I4862A: 77.6 ± 1.4; L4865A: 52.3 ± 3.4; I4867A: 42.0 ± 1.8; F4870A: 62.4 ± 9.1 and L4873A: 93.5 ± 5.6 ± 1 s−1, p<0.05, n=5-8). Our data support the proposal that blockade by TAAs is stabilised by interactions with hydrophobic residues of TM10 lining the cytosolic cavity of the RyR2 pore. Welch W, Rheault S, West DJ, Williams AJ. A model of the putative pore region of the cardiac ryanodine receptor channel. Biophys J. 2004 October 1, 2004;87(4):2335-51. Mead-Savery FC, Wang R, Tanna-Tolan B, Chen SR, Welch W, Williams AJ. Changes in negative charge at the luminal mouth of the pore altered ion handling and gating in the cardiac ryanodine-receptor. Biophys J. 2009 February 18, 2009;96(4):1374-87. Tinker A, Lindsay AR, Williams AJ. Large tetraethyl ammonium cations produce a reduced conductance state in the sheep cardiac sarcoplasmic reticulum Ca(2+)-release channel. Biophys J. 1992 May 1, 1992;61(3):1123-32

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PC264
Identification of gating mutations in the TREK-1 K2P potassium channel by functional complementation in K+ uptake deficient yeast
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TREK-1 is a member of the K2P family of potassium channels. These channels appear to have a unique gating mechanism compared to other types of K+ channel and this may be a reflection of their overall asymmetric structure. In order to address which domains of the channel may be important for channel gating we took advantage of an unbiased random mutagenesis approach which selects for activatory mutations by complementation in a K+ auxotrophic strain of yeast (SCY1528). Wild-type TREK-1 did not complement the growth of this strain on low [K+] media. However, screening a randomly mutated TREK-1 library yielded a number of mutations which robustly complemented growth. One of the mutants has been identified previously through its effects on pHi-gating (Glu-321). However many other novel mutations were also identified. Intriguingly, the majority of these mutations were located in the TM5 and/or close to the selectivity filter of TREK-1. Electrophysiological analysis of these mutants expressed in Xenopus oocytes demonstrates dramatic effects on TREK channel gating by intracellular pH. The results also point towards a dominant role for the inactivation gate (i.e. selectivity filter) and TM4 in TREK-1 channel gating.

PC263
Mice expressing a human K_{ATP} channel mutation have altered channel ATP sensitivity, but no cardiac abnormalities
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Patients with severe gain-of-function mutations in Kir6.2 (KCNJ11), the pore-forming subunit of the ATP-sensitive potassium (K_{ATP}) channel, have neonatal diabetes, muscle fluidity, and mental and motor developmental delay; a condition known as iDEND syndrome. Despite the fact that Kir6.2 forms the pore of the cardiac KATP channel and is abundantly expressed in the heart, the patients show no obvious cardiac symptoms. We used a mouse model of iDEND syndrome to determine if the lack of cardiac symptoms is because iDEND mutations do not alter ATP inhibition of the cardiac channel. We studied mice in which the most common iDEND mutation (Kir6.2-V59M) was targeted to cardiac muscle using Cre-lox technology (m-V59M mice). Quantitative rtPCR revealed that wild-type and mutant mRNAs were expressed at about the same level in the heart of m-V59M mice, indicating that m-V59M mice simulate the heterozygous state of the patients. Patch-clamp studies of isolated cardiac myocytes revealed a marked reduction in the sensitivity of the K_{ATP} channel in m-V59M mice to MgATP inhibition (IC_{50} = 62 μM compared to 13 μM for littermate controls). In vivo cme MRI of mice anesthetized with 1.5% isoflurane in O2 identified no gross morphological differences and no differences in heart rate, end diastolic volume, end systolic volume, stroke volume, ejection fraction, cardiac output or wall thickening between m-V59M and control hearts, either under resting conditions or under dobutamine stress. In summary, the common iDEND mutation Kir6.2-V59M decreases ATP block of cardiac KATP channels but is without effect on heart function. This suggests that metabolic changes fail to open the mutated channel to an extent that affects function. In contrast, when the Kir6.2-V59M mutation is expressed in pancreatic beta-cells, mice fail to secrete insulin. It is likely these different effects on cell function result from differences in the SUR subunit of the channel (SUR1 in beta-cells; SUR2A in heart).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Simulation analysis of Ca\textsuperscript{2+}-dependent inhibition dynamics of L-type Ca\textsuperscript{2+} current of pulmonary vein cardiomyocytes of rabbit

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Ca\textsuperscript{2+} dependent inactivation (CDI) of L-type Ca\textsuperscript{2+} current (ICaL) is well known phenomena. In a recent report, Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) greatly affected the inactivation kinetics of L-type Ca\textsuperscript{2+} current in rat ventricular myocytes (a release dependent inactivation, RDI, Zahradnikova et al., 2004). We enzymatically isolated single cardiac myocytes in pulmonary vein of rabbit and applied the whole cell patch clamping technique. All procedures were accorded with national legislation. We found CDI was greatly affected by the change of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (NCX). Based on the cardiac myocyte model which could reproduce the subsarcolemmal Ca\textsuperscript{2+} dynamics (Leem et al., 2006), we reproduced CDI dynamics. We could analyze the Ca\textsuperscript{2+} binding dynamics on Cal, the subsarcolemmal Ca\textsuperscript{2+} change, and the effect of NCX and sarcoplasmic reticular (SR) Ca\textsuperscript{2+} release on CDI. The simulation results well reproduced the CDI phenomena. To reproduce CDI dynamics, it was postulated that two different Ca\textsuperscript{2+} binding sites, fast and slow, were existed and the Cal had four of each site. The simulation also reproduced the CDI dynamics in the presence and absence of NCX and the effect of SR dysfunction on CDI. In the experiment, the removal of NCX greatly increased CDI phenomena and considerably slowed the recovery from CDI. From these results, we initially thought NCX greatly modulated subsarcolemmal Ca\textsuperscript{2+} concentration and therefore, CDI was greatly affected. However, in the simulation, the NCX mainly affected the refilling extent of SR Ca\textsuperscript{2+}, and affected the extent of Ca\textsuperscript{2+} release from SR which caused CDI. In conclusion, we successfully reproduced CDI phenomena in various conditions and Ca\textsuperscript{2+} release from SR is the most important factor to induce CDI. NCX could greatly modulate SR Ca\textsuperscript{2+} refilling extent and indirectly affect CDI.


This work was supported by the grant from NRF (No. 2009-0076234)

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Preventive effect of mitochondrial substrates and calmodulin on L-type Ca\textsuperscript{2+} current in atrial cardiac myocytes of rabbit

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L-type Ca\textsuperscript{2+} current (ICaL) is essential component to induce excitation-contraction coupling in cardiac myocytes. Therefore, ICaL is a major target of various signaling pathways. One of the difficulty to study on ICaL is run-down phenomena. In this study, we would like to see the role of mitochondria as a ATP factory. We enzymatically isolated single atrial myocytes of rabbit. All procedures were accorded with national legislation. It is well known that ICaL requires MgATP and calmodulin to maintain their function. However, even though 5 mM ATP is added in the pipette solution, ICaL became run-down and eventually disappeared. In this report, we tested the effect of mitochondrial substrates and calmodulin on ICaL maintenance. When we added pyruvate or malate, ICaL was maintained longer than that without the substrates. When we added malate and calmoduline together, ICaL was maintained much longer, even more than 1 hour. These results suggested that mitochondrial substrates were essential to support ICaL in sarcolemma and calmodulin was strong supportive effect on ICaL maintenance. Glucose was continuously present in the experimental conditions, however, only presence of glucose is not enough to support ICaL in whole cell patch clamp conditions. 5 mM ATP was continuously present but it could not prevent ICaL rundown which meant that 5 mM ATP in pipette solution was not enough to maintain ICaL in sarcolemma. We tested NADH level in the presence of mitochondrial substrates such as pyruvate and malate and compared the maximum NADH level obtained in the presence of rotenone. Only presence of pyruvate could achieve 50% of the maximum NADH. In the presence of both malate and pyruvate, NADH level could reach more than 80% of the maximum NADH. These results suggested that the addition of the mitochondrial substrates was essential to maintain intracellular ATP level in whole cell patch conditions.

This work was supported by the grant from MWHF (No. A084776)

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Characterization of a TRPM7-like cation current in human atrial cardiomyocytes

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Transient receptor potential melastatin 7 (TRPM7) channels, which is known as Mg\textsuperscript{2+}-inhibitable cation current (MIC) or Mg nucleotide-regulated metal ion current (MagNuM) have recently been discovered to play important roles in Mg\textsuperscript{2+} and Ca\textsuperscript{2+} homeostasis, which is critical to both human health and
cell viability. However, most of these data were obtained using the heterologous expression systems, very few data in the heart cells of different animals, while in native human cardiomyocytes has not yet been described. We aimed to identify the presence of TRPM7-like current in human atrial cardiomyocytes and evaluate the origin of the permeability of the channel and its regulation using the whole-cell voltage clamp technique. Atrial myocytes were isolated from right atrial appendages from 52 adult patients in sinus rhythm (SR) with and without ischaemic heart disease, with coronary artery disease or valve diseases. We demonstrate that under voltage clamp conditions using voltage ramps between -120 mV and +80 mV, an outward-rectifying current at positive potentials and small inward current at negative potentials, was present in cells dialyzed with zero [Mg2+]o, i.e. current increased from 1.67±0.07 to 3.73±0.20 pA/pF at +80 mV, and from –0.26±0.02 to -0.50±0.03 pA/pF at -120 mV, (n=81, p<0.001). The current was suppressed by raising [Mg2+]o and was absent in cells dialyzed with physiological [Mg2+]o, indicating that it was due to the Mg2+-sensitive, TRPM7-like current. Extracellular application of Gd3+, 2-APB and spermine (at 100 μM), known to block TRP channels, decreased the peak density of TRPM7-like current outward component almost completely. It is interesting that additional large current was induced in divalent free solutions, when cells were dialysed with physiological or high intracellular free Mg2+ (up to 10 mM) or MgATP (5 mM) concentration, which did not protected the spontaneous run-up in a concentration dependent manner. This was due not to the movement of monovalent ions via L-type Ca2+-channels because that current was resistant to 100 μM nifedipine. Moreover, the difference current obtained on the ascending limb of the 4 sec ramp was the same or nearly the same as on the descending limb, that implies that the current we measure is TRPM7-like. In addition, we established that the density of activated TRPM7-like current was related to the clinical history of the donor, i.e. in cells from patients with the ischaemic heart disease the outward current was higher (3.92±0.32 pA/pF, n=57, p<0.001) as compare with those without ischaemia (3.19±0.32 pA/pF, n=20, p<0.001). In conclusion: to our knowledge, this is the first study, which describes TRPM7-like current in native human atrial cardiomyocytes that operates under physiological/pathophysiological conditions.

Work was supported by Research Council of Lithuania grant No.MIP-78/2010

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**PC269**

**Coupling of the Voltage Sensing Domain to the Pore Domain in the hERG K⁺ Potassium Channel**

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The gating of voltage gated potassium (K) channels is a fundamental process for regulating cellular excitability. Voltage gated K⁺ channels are tetramers with each subunit containing six transmembrane spanning helices (S1 – S6). S1 – S4 forms the voltage sensing domain (VSD) and is joined via the S4-S5 linker (S4S5L) to the pore of the channel formed by S5-S6. How the VSD couples to the pore domain is not well understood, but is thought to involve interactions between the S4-S5 linker and S6. hERG channel gating is characterised by rapid inactivation gating and slow deactivation gating and these processes are important for regulating the physiological time course of this current during the cardiac action potential. When the Val at position 659, in the Cterminal end of the S6, was mutated to Ala during an Ala scan of S6 it profoundly slowed deactiva-
tion. Our aim was to investigate the molecular basis for the slow deactivation of V659A. Site-directed mutagenesis was used to mutate Val659 to a range of amino acids and effects on gating were studied by two-electrode voltage clamp in Xenopus oocytes. Mutation to bulky hydrophobic residues like Trp, Ile & Phe had little effect on deactivation. However, mutation to smaller residues like Cys & Ala slowed deactivation (τ's at -140mV, WT 22±13ms, 659ile 202±17ms, 659Ala 3181±156ms, n=6). V659C would not deactivate even at -200mV indicating that the pore was no longer coupled to the VSD. The crystal structure of Kv1.2 shows the S4S5L passes across the S6 close to the activation gate. To test if Val659 was in close proximity to the S4S5L we investigated if the oxidising agent tert-Butyl hydroperoxide (2mM) could facilitate disulphide bonds between 659Cys and Cys residues substituted into different positions on the S4S5L. We ran a current-voltage (I-V) protocol, holding at -120mV and stepping to test potentials from -90mV to +30mV then back to the holding potential in the presence of the oxidising agent. The I-V relationship for the double mutant V659C:E544C became linear and there was a large increase in instantaneous current (the current 5ms after stepping to the test potential) suggesting that the channel was closed but not open with the addition of the oxidising agent. This effect was not reversible on switching back to the control solution & was inhibited by pre-treating the cells with a reducing agent (10 mM Dithiothreitol). We investigated if this effect was happening when the channel was in the open or closed state by holding the channel closed at -120mV or open at +40mV and washing on the oxidising agent for 5 minutes and measuring the instantaneous current at +20mV. In the closed state current increased 18±6% but in the open state the current increased 78±5% (n=4). Our results suggest that Val659 couples to the S4-S5L in the open state forming an interaction that is required for closing the activation gate of hERG.

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PC270

Description of the gating behaviour of purified human cardiac ryanodine receptor (hRyR2) by kinetic modelling and burst analysis under minimal conditions

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Rhythmic contraction of cardiac myocytes is maintained by precisely controlled Ca2+ efflux from intracellular stores mediated by the cardiac ryanodine receptor (RyR2)1. Mutations in RyR2 can cause channel instability leading to perturbed Ca2+ release that can trigger arrhythmias2. RyR2-dependent ventricular tachyarrhythmia is an important cause of sudden cardiac death, the mechanistic basis of which remains unclear. Most investigations of RyR2 single channel function have focussed on the secondary effects of mutation on single channel open probability (Po) via its modulation by regulatory proteins and cellular processes without emphasis on mutation-dependent effects on the gating behaviour of the channel per se. In this investigation we provide key novel mechanistic insights into the physical reality of RyR2 gating revealed by new experimental and analytical approaches. We have examined in detail the single channel gating kinetics of the purified hRyR2 when activated by cytosolic Ca2+ ([Ca2+]cyt) in a stringently controlled environment where the modulatory influence of factors external to the channel were minimised. Ca2+ activation of single purified recombinant hRyR2 channels was monitored in planar lipid bilayers3,4 where the luminal Ca2+ was buffered at 50 nM while the [Ca2+]cyt was stringently controlled using a cocktail of buffers to achieve an activating free [Ca2+]cyt range of nominally zero-500 μM. Single channel data were accurately analysed using hidden markov model (HMM) based algorithms in the QuB suite of analysis programs (www.qub.buffalo.edu). The resultant sigmoidal dose-response curve gave an EC50 of 1.45±0.44 μM (n=10) with Po saturating at ~10 μM Ca2+. The gating model generated describes the kinetic behaviour using a minimum of four open and three closed states and incorporates constitutive (unliganded) gating activity (Po: 1.09±0.5x10⁻⁵, open time: 0.17±0.06 ms; n=10) and reveals ligand-independent fast flicker closings transitions (τ: 0.38±0.03 ms, n=4) suggestive of gating events similar to Ctype inactivation in K+ channels5. The kinetics also suggest an inhibitory role of [Ca2+]cyt beyond 1 μM where the backward ligand-dependent transitions were perturbed. Simulation using the putative gating models generated data similar to the actual and served to further validate the model. Novel detailed burst analysis of hRyR2 elucidates its ligand-bound gating kinetics where burst length increases and interburst interval decreases with increasing [Ca2+]cyt.

This proposed model will serve as a benchmark against which the effects of disease causing mutations in hRyR2 can be studied, as well as the influence of physiological modulators and potentially therapeutic compounds capable of stabilising mutant RyR2 channel function.


The research is supported by BHF and Cardiff University.

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PC271

Studies on IKr Channel Blockade in Human Cardiomyocytes as a Cause of Arrhythmias

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According to guidelines from the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) all new drugs both from the point of teratogenicity and the adult risk of sudden death should be tested regarding proarrhythmic
Ca\textsuperscript{2+} changes with ethyleneglycol-bis-(\beta-aminoethylether)\-
\textsuperscript{-}N,N,N',N'-tetraacetic acid. The frequency of the oscillating cur-
rents was transiently remained at the holding potential, -40 mV as
(1 sec duration, 30 sec intervals) and the oscillating current
was increased as intracellular [Na\textsuperscript{+}] increased. One of the
interesting features was that those currents became activated
by intracellular Ca\textsuperscript{2+} loading and IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release.

This work was supported by the grant from NRF (No. 2010-
1024)

Where applicable, the authors confirm that the experiments
described here conform with The Physiological Society ethical
requirements.

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**PC273**

L-type calcium modulates the pro-arrhythmic response to
dofetilide in human: a simulation study

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**Introduction:** Dofetilide is a class III drug whose use needs to be monitored
due to the possible occurrence of Torsades de Pointes (TdP). The mechanisms
are unclear but research suggests that both early after depolarizations (EADs)
and increased dispersion of the action potential duration (APD) might be involved.
Importantly, drug-induced TdP is more common in women, possibly
due to higher L-type calcium current (I\textsubscript{CaL}) levels.

Our goal is to investigate pro-arrhythmic mechanisms of
dofetilide in human using computer simulations. We will assess
the effect of dofetilide on the APD dispersion and prolonga-
tion, EADs formation and the intracellular calcium concentra-
tion ([Ca\textsubscript{2+}]) for various doses and I\textsubscript{CaL} levels.

**Methods:** A transmural human ventricular model was constructed includ-
ing endocardial, midmyocardial and epicardial cells. We use
the monodomain model to describe the electrical wave in the
tissue and the Grandi et al model (1) to describe the mem-
brane kinetics. Dofetilide-induced HERG block formulation was
included to the ionic model following (2). The computational
domain was simulated with different frequencies 2Hz, 1Hz and
0.5Hz at the endocardium and the electrophysiological
activity was simulated for various dofetilide doses [D]=0 to
120 nM and for I\textsubscript{CaL} from fCaL=100 to 140% of control. All
the results that we show correspond to the simulation with
1Hz frequency

**Results:** In agreement with experimental recordings (3), in control
(I\textsubscript{CaL}=100\%), dofetilide increases APD by 20% and APD dis-
ersion by 20-30ms but no EADs are observed.
EADs appearance depends on both parameters [D] and I\textsubscript{CaL}.
Fixing one and increasing the other results in EADs appear-
ance. Panel C (resp D) shows the role of the dose dofetilide
(resp I\textsubscript{CaL}) on the EADs genesis.
EADs result in long APD of up to 1200 ms (panel A) and impor-
tantly, increased APD dispersion of up to 300 ms (panel B) due
to transmural differences in EADs durations.
EADs are also observed in the pseudo-ECG for different
dofetilide doses (panel E) and levels of I\textsubscript{CaL} (panel F). Their

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**PC272**

IP\textsubscript{3} sensitive, Ca\textsuperscript{2+}-dependent oscillating currents in cardiac
myocytes of pulmonary vein of rabbit

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Atrial fibrillation (AF) is the most common kind of arrhyth-
mia, however, its etiology is still obscure. Pulmonary veins (PVs)
were found to be important ectopic foci for the initiation and
maintenance of AF. Previous report suggested that Ca\textsuperscript{2+}
dysregulation induced by ryanodine and rapid pacing in PVs caused the arrhythmogenic activity (Honjo et al., 2003). We enzymatically isolated single cardiac myocytes in pulmonary vein of rabbit. All procedures were accorded with national legislation. Using whole cell voltage clamping tech-
nique, we found oscillating outward currents activated by step
depolarization pulses. The oscillating outward currents were
CI-dependent and abolished by the removal of intracellular
Ca\textsuperscript{2+} changes with ethyleneglycol-bis-(\beta-aminoethylether)\-
N,N,N',N'-tetraacetic acid or 1,2-bis(O-aminophenoxy)ethane-
N,N',N'-tetraacetic acid. The frequency of the oscillating cur-
rent was increased as intracellular [Na\textsuperscript{+}] increased. One of the
interesting features was that those currents became activated
by serial application of step depolarization pulses to +60 mV
(1 sec duration, 30 sec intervals) and the oscillating current
was transiently remained at the holding potential, -40 mV as
an inward current. When depolarizing step pulses were applied
repeatedly, the remaining time of the oscillating currents at
the holding potential became longer and finally continuously
activated. Those inward oscillating currents certainly act as a
depolarization force and may cause arrhythmogenic activity.
The oscillating currents were not observed in atrial cardiac
myocytes in atrial appendage. We tested IP\textsubscript{3}, dependency of
the oscillating currents. We found IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} release
blockers such as heparin and 2-aminoethoxydiphenyl borate
(2-APB) blocked the oscillating currents. And also phospholipase
C inhibitor, U73122, abolished the oscillating current acti-
vation. Those results suggested that the oscillating currents
may be dependent on [IP\textsubscript{3}], oscillation. In conclusion, the car-
diac myocytes in PV can initiate arrhythmogenic Ca\textsuperscript{2+}-depend-
ent oscillating currents activated by intracellular Ca\textsuperscript{2+} loading
and IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release.

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200P
appearance could be explained by a spontaneous calcium release. In fact plotting the intra-cellular calcium concentration shows that EADs appearance in panel E (resp B) is synchronized with an intra-cellular calcium release panel G (resp H). The magnitude of the EADs in the pseudo-ECGs is also scalable with the released calcium concentrations.

Conclusions:
ICaL is a key modulator of dofetilide-induced EADs in human ventricular tissue. A combination of high dofetilide doses and L-type calcium current is responsible for the EADs appearance. Importantly, EADs cause a significant increase in APD dispersion, which might provide the substrate for the establishment of reentry and TdP.

Figure 1. Role of dofetilide and L-type calcium current on the APD and APD dispersion prolongations.

Figure 2. Role of dofetilide and L-type calcium current on the EADs appearance in the ECG and the intracellular calcium release.


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PC274

The sGC activator BAY58-2667 protects against sterile inflammatory shock in mice
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Reactive oxygen species can oxidize the ferric-heme prosthetic group of soluble guanylate cyclase (sGC) resulting in the formation of apo-sGC that is nitric oxide (NO)-insensitive and is proteolytically degraded. The sGC activator BAY58-2667 (1) can bind apo-sGC, thereby stabilizing it and increasing cyclic GMP production in a heme- and NO-independent manner. Starting from the observation that the hypoxic NO-donor nitrite (NO₂⁻) (2) can sGCdependently protect against sterile inflammatory shock (3), we decided to test BAY58-2667 in a murine model of endotoxic shock and found that it can protect against morbidity and mortality, as evidenced by a rapid return of normal resting heart rate (HR), heart rate variability (HRV), and mean arterial pressure (MAP). Female C57BL/6 mice were injected IV with LPS (10 mg/kg) to induce endotoxic shock. Treatment was either vehicle or BAY58-2667 (100 μg/kg) IV, given +3h or +8h after challenge. Rectal temperature was used as a read-out of morbidity and was improved 30h after LPS in the +8h (33.4 °C ± 0.48; n = 4) group compared to controls (27.2 °C ± 0.43, n = 4), whereas the +3h treatment exacerbated morbidity (100% mortality 30h after LPS, n = 5). Mortality 60h after LPS challenge was also lower in the +8h group compared to control and +3h group: controls (100% mortality, n = 9) versus +3h (100% mortality, n = 5) and +8h group (32.5% mortality, n = 9). Values are means ± SEM. HR, MAP, activity, and core temperature were determined using PA-C10 and ETA-F20 implantable transmitters (Data Sciences International). Female C57BL/6 mice were anesthetized using inhaled isoflurane (induction 4%, maintenance 2%). Analgesia was started 24h before the procedure until complete recovery (Ibuprofen, 200 μg/ml in drink). Following baseline measurement, implanted mice received a bolus IV injection of LPS (10 mg/kg) to induce endotoxic shock. Afterwards they were treated with vehicle or BAY58-2667, +3h or +8h after LPS. Infusion of LPS was associated with an immediate loss of activity, and progressive decline in HR, MAP and core temperature. The +3h group (n = 2) was again sensitized and did not recover, whereas the HR and MAP in the +8h group was stabilized already 24h after LPS, and HRV and circadian rhythms returned to normal 30h after LPS (n = 2). One control mouse died 50h after challenge. The second control survived but it took up to 5 days for HR and MAP to recover fully as compared to only 30-35h for the +8h treated animal. We conclude that reactivation of sGC in regions exposed to high levels of oxidative stress, as occurs during ischemia, can restore tissue metabolic homeostasis. Whether or not this (1) occurs at the level of the microcirculation of vital organs; (2) is a consequence of a direct or indirect beneficial effect on cardiac function; (3) or is a combination of both, remains to be determined.


Chronic intermittent hypoxia-induced respiratory muscle fatigue is mediated by reactive oxygen species

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Obstructive sleep apnoea syndrome (OSAS) is characterized by recurrent collapse of the upper airway during sleep. The disorder is very common and is associated with significant cardiovascular morbidity. Respiratory muscle dysfunction is documented in OSAS patients and it is speculated that this, and other key morbidities, may be a result of oxidative stress which is secondary to the repeated hypoxia/re-oxygenation events characteristic of the condition due to apnoea. We sought to explore the effects of chronic intermittent hypoxia (CIH) on respiratory muscle function in a rodent model 1, and to test the efficacy of antioxidant agents in preventing CIH-induced respiratory muscle impairment.

Adult male Wistar rats were exposed to CIH (n=8) consisting of 90s normoxia/90s hypoxia [5% oxygen at the nadir; SaO2 ~ 80%] for 8h/day or to sham treatment (air/air, n=8) for 2 weeks. Three additional groups of CIH-treated rats had free access to water containing N-acetyl cysteine (1% NAC, n=8), tempol (1mM, n=8) or apocynin (2mM, n=8). Following gas treatments, diaphragm and sternohyoid (pharyngeal dilator) muscle contractile and endurance properties were examined in vitro at 35°C. Additionally, muscle was snap frozen and stored for structural analysis.

CIH increased sternohyoid muscle fatigue (63±7 vs. 47±7%, sham vs. CIH, mean±SEM, % of initial force after 2 min of repeated stimulation, p=0.08, ANOVA), but apocynin treatment during CIH exposure only partially recovered muscle endurance (56±5%). All three drugs reversed the deleterious effects of CIH on diaphragm endurance (52±5 vs. 38±3, sham vs. CIH, p=0.05); 62±6%, 67±6% and 58±6% NAC, tempol and apocynin respectively). There was no significant effect of CIH treatment on respiratory muscle MHC fibre type, relative area of fibres expressing SERCA 1 and 2 protein or Na+-K+-ATPase pump content.

We conclude that the deleterious effect of CIH on respiratory muscle endurance is due to oxidative stress since, for the most part, antioxidants reversed CIH-induced respiratory muscle fatigue. Pharmacological blockade of the superoxide generating NADPH oxidase (NOX) enzyme with apocynin improved diaphragm muscle endurance, suggesting that cytosolic oxidases may be a source of increased ROS production in CIH. Our results suggest that respiratory muscle dysfunction in OSAS may be the result of oxidative stress, and as such antioxidant treatment could prove a useful adjunct therapy in the treatment of OSAS.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Maternal fructose and/or salt consumption programs cardiovascular hypersensitivity in the offspring

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Introduction: Maternal diet through pregnancy impacts on the offspring’s susceptibility to later cardiovascular disease (CVD). A typical modern Western diet is high in fructose and salt and when such a diet is consumed through pregnancy and lactation, in a rat model, the salt-exposed offspring have marked sex-specific differences in mean arterial blood pressure. In this study, we have further assessed the male and female offspring in terms of their cardiovascular responses to further, short-term intake of fructose or salt.

Methods: 21 virgin Sprague-Dawley rats were randomly divided into 4 dietary groups; 1) control diet (CD, n=6) fed purified chow and tap water, 2) Salt diet (SD, n=5) fed purified chow + 4% NaCl, 3) Fructose diet (FD, n=5) fed purified chow and 10% fructose in tap water and 4) Fructose & Salt diet (FSD, n=5), fed 4% NaCl purified chow with 10% fructose in tap water. Animals were fed the diets ad libitum for 28 days prior to conception, throughout gestation to weaning, whereupon all offspring received standard chow diet until 9 weeks of age when they were implanted with radiotelemetry probes (under fentanyl citrate and medetomidine hydrochloride, 300 μg kg⁻¹ of each i.p) for cardiovascular recording. After 7 days recovery, offspring were exposed to 1) isolation-induced anxiety for 24h (i.e. sibling removed from cage), 2) salt diet (4% NaCl) for 5 days and 3) 10% fructose solution for 5 days with a 7 day wash-out period between challenges. Cardiovascular variables were recorded at scheduled intervals throughout the challenges and data analysed by General Linear Mixed Models (Genstat v13, VSNI, UK).

Results: Mean arterial pressure (mm Hg) of offspring was (males: CD, 106; FD, 110; SD, 121; FSD, 110 ±3 [s.e.d]; females: CD, 112; FD, 110; SD, 102; FSD, 109 ±4 [s.e.d]). With anxiety, male but not female salt-exposed offspring had a significantly steeper relationship between paired values for mean arterial blood pressure and heart rate (e.g. calculated slopes were CD, 3.26 (3.02-3.49) vs. SD, 5.36 (5.17-5.55); mean, 95% confidence interval). Postnatal exposure to salt had little effect on offspring cardiovascular variables but exposure to fructose revealed increased sensitivity in prenatal fructose-exposed offspring (see Figure).

Conclusions: The data suggest that increased maternal intake of sugar sweetened beverage or of salt has significant effects on the cardiovascular system of the offspring, including altered resting levels of blood pressure but also hypersensitivity to an anxiety-related stimulus (male prenatally salt-exposed offspring only) and, in terms of pressor activity, to further fructose intake. The data therefore lend support to the recent cautionary statements regarding increased fructose and/or salt intake in the Western diet.
The impact of NAD(P)H oxidase on the regulation of renal hemodynamics

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Endogenous autocrine and paracrine factors which regulate blood flow through the kidney which may be exerted to different degrees in the cortex and medulla. Superoxide anions, generated by enzymes such as NAD (P) H oxidase, have the potential of modulating the tone of the renal microvasculature but their actions are normally limited as a result of scavenging by superoxide dismutase. The aim of this study was to investigate the contribution of the superoxide anions in the regulation of cortical and medullary blood perfusions generated by NADPH oxidase. This was done by blocking superoxide dismutase activity in rats where NADPH oxidase intact or blocked.

Two groups (n=9) of male Wistar rats (210-292g) were used with one group maintained on Apocynin (454mg/l) in drinking water for 7-10 days. Anesthesia was induced with 1ml ip chloralose/urethane (16.5/250mg/ml). The right femoral vein was cannulated for infusion of saline at 3ml/h containing inulin. The right and left kidneys were exposed, their ureters cannulated for urine collection and a small cannula inserted 4.0-4.5 mm into the cortico-medullary border of the left kidney for the infusion of saline and BK at a concentration of 3x10^-6 or 6x10^-6g/l at 1 ml/h. After 1.5h, 20 min clearances were taken, two before and two 15 min after the start of the BK infusions. Two groups were studied, one in which the renal sympathetic nerves to the left kidney were intact (n=7), and a second (n=6) in which they were disrupted by bathing with 10% phenol. The rats were killed at the end of the experiment. Data, means ±SEM were subjected to ANOVA and significance taken at P<0.05.

Intrarenal infusion of both doses of BK had no effect on MAP, at 107±6mmHg or glomerular filtration rate (GFR) in either left or right kidney, at 2.58±0.50 and 3.53±0.97ml/min/kg, respectively. The lower dose of BK had no effect on fractional sodium excretion (FENa) in either left or right kidneys, at 1.07±0.26% and 0.93±0.37%, respectively, and while the higher dose had little effect on FENa in the left kidney it was decreased (P<0.05) by 38% in the right kidney. Denervation of the left kidney had no effect on MAP or GFR but increased (P<0.05) FENa by 33% from that kidney. FENa did not change in either left or right kidney, at 2.58±0.50 and 3.53±0.97ml/min/kg, respectively. The lower dose of BK had no effect on fractional sodium excretion (FENa) in either left or right kidneys, at 1.07±0.26% and 0.93±0.37%, respectively, and while the higher dose had little effect on FENa in the left kidney it was decreased (P<0.05) by 38% in the right kidney. Denervation of the left kidney had no effect on MAP or GFR but increased (P<0.05) FENa by 33% from that kidney. FENa did not change in either kidney when BK at low or high dose was infused into the left kidney.

These findings showed that infusion of BK into the renal interstitium whilst having minimal effects on renal haemodynamics or fluid handling of that kidney, elicited a contra-lateral anti-
The role of Angiotensin (1-7) in the regulation of renal haemodynamics

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The Renin Angiotensin System (RAS) is a powerful endogenous hormonal cascade that is involved in the maintenance of renal, cardiovascular and extracellular fluid volume homeostasis via the potent vasoconstrictor, Angiotensin II (Ang II). Recently, it has emerged that a second Angiotensin Converting Enzyme (ACE) isofrom, ACE 2 acts upon Ang II to generate Ang (1-7), a vasodilator with natriuretic and diuretic actions at the kidney that directly oppose those of Ang II. Currently, the precise way in which Ang (1-7) influences renal hemodynamics is unclear. Thus, the aim of this study was to determine whether Ang (1-7) would increase renal blood flow (RBF) and whether these affects were solely dependent upon Mas receptor activation or whether simultaneous Ang II induced AT 1 receptor stimulation was required.

Groups of (n=4-8) male Wistar rats were anaesthetized (i.p. 1.2ml chloralose/urethane, 16.5/250mg/ml) and prepared for measurement of mean arterial blood pressure (MAP) and RBF, the intravenous infusion of saline at 3ml/h and the corticomедullary infusion of drugs at 1.0ml/h. Increasing doses of Ang (1-7) (9x10^{-10} M; 3x10^{-9}M; 9x10^{-9}M; 2.7x10^{-10} M) were infused into group 1 rats in a random order. Losartan (AT1 antagonist: 3x10^{-6}M) and A-779 (Mas antagonist: 3X10^{-7}M) were co-infused into Group 2 and 3 rats, respectively along with previously mentioned doses of Ang (1-7). Anaesthetic requirements. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC279

PC280

Chronic intermittent hypoxia induces respiratory muscle weakness in neonatal rats which persists into early adult life

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The respiratory system is subject to considerable developmental plasticity and perturbations during vulnerable periods of early life can induce persistent maladaptive changes. Exposure to intermittent bouts of hypoxia (IH) is a common consequence of several neonatal respiratory disorders - such as apnoea of prematurity. Oxidative stress is implicated in hypoxia-induced muscle dysfunction in disease states. In this study we sought to investigate the effects of chronic IH (CIH) during early development on respiratory muscle structure and function, and to determine if CIH exposure during early neonatal life has long-lasting effects on respiratory muscle function. Litters of Wistar rats, together with their dams, were placed from birth in hypoxia chambers. The CIH litters received alternating cycles of 90 sec hypoxia (reaching 5% O2 at the nadir) and 210 sec normoxia for 8hr/day for 7 days. Sham litters were exposed to circulating normoxic gas for 7 days. After gas treatments, Sham (n=8) and CIH (n=8) sternohyoid (pharyngeal dilator) muscle functional properties were examined in vitro. Littermates from sham and CIH groups were returned to normoxia for 7 or 21 days, after which functional studies were performed (n=8 all groups). Adult sham (n=8) and CIH (n=8) Wistar rats were also studied. Sternohyoid muscle fibre type and size was evaluated by myosin heavy chain (MHC) immunofluorescence. Lipid peroxidation in muscle was evaluated by TBARS assay.

CIH caused a significant depression of sternohyoid force at post-natal day (PD) 7; peak force was 4.3 ±0.8 N/cm² vs. 1.6±0.3 N/cm²; control vs. CIH, Student’s t test, P<0.01. There was no major structural reorganization in CIH-treated PD7 muscle, though the areal density of MHC developmental fibres was significantly decreased, whereas MHC I (slow) fibres was significantly increased compared to sham muscles. In comparison CIH had no effect on adult sternohyoid muscle force. The negative inotropic effect of CIH on sternohyoid force persisted 7 (8.6±1.0 N/cm² vs. 6.0±1.1 N/cm²) and 21 (12.9±2.7 N/cm² vs. 11.0±0.6 N/cm²) days after CIH treatment (P<0.001, two-way ANOVA). Contrary to expectation, there was no evidence of increased lipid peroxidation in CIH treated muscles.

CIH causes upper airway dilator muscle impairment in neonatal but not adult rats. Respiratory muscle weakness in CIH-treated neonates persists into early adult life despite a return to normoxia. Impaired pharyngeal dilator muscle function could have deleterious consequences for the maintenance of upper airway patency in vivo. We speculate that CIH-induced respiratory muscle remodelling could exacerbate and perpetuate neonatal respiratory disorders characterized by recurrent hypoxia.

Supported by the Health Research Board Ireland and the Irish Research Council for Science, Engineering and Technology.
Protective effect of hypercapnia on warm ischemia induced free radical damage of lungs retrieved from non-heart-beating donors

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Lungs retrieved from non-heart-beating donors (NHBD) might alleviate the shortage of suitable organs for transplantation. The critical point is the preservation of lungs during warm ischemia, when damage is caused by free radicals [1-2]. We have demonstrated previously that ventilation during warm ischemia worsens lung free radical damage. Also, pre arrest administration of radical scavenger, tempol, reduces warm ischemic damage of lung functions [3]. Hypercapnia has protective effect on free radical damage [4-5]. In present study we investigated a possible protective effect of ventilation using hypercapnic gas mixture during warm ischemia on free radical damage in lungs retrieved from non-heart-beating donors. Four groups (n = 8) of Wistar male rats were used – 3 experimental and 1 control group. All the experimental groups underwent the protocol of NHBD lung harvesting – the tracheal canula was introduced under thiopental anaesthesia (50mg/kg, administered intraperitoneally) and the animals were killed by overdosing of sodium thiopental (250mg/kg, administered intraperitoneally). Then the animals were kept in room temperature for 60 minutes – warm ischemia. The groups were: V – ventilated during warm ischemia with room air; H – ventilated during warm ischemia with hypercapnic gas mixture (7% CO2, 21% O2, 72% N2); N – non ventilated during warm ischemia; C- controls, lungs isolated immediately after thiopental anaesthesia without warm ischemia. Then in isolated rat lungs we measured perfusion pressure, weight, and arteriovenous difference in oxygen partial pressure in time intervals of 30, 60, 90 and 120 minutes after reperfusion. For statistical evaluation we used ANOVA for repeated measures, Games/Howell post hoc test, p ≤ 0.05; values are presented as means ± SEM.

We found that ventilation with the room-air caused severe pulmonary oedema within 30 minutes after reperfusion in 3 of 8 animals and also in the rest of V group we found significant increase of lung weight (0; 0.23±0.15; 0.43±0.19; 0.53±0.22) compared to controls (C; 0; 0.03±0.07; 0.08±0.1; 0.04±0.13; p≤0.05) or non-ventilated animals, (N; 0; 0.05±0.09; 0.04±0.14; -0.06±0.14; p≤0.05). In contrast to this after ventilation with hypercapnic gas mixture (H) we did not observe any signs of pulmonary oedema and the lung weight gain (0; 0.05±0.01; 0.12±0.02; 0.2±0.04) did not differ compared to non-ventilated (N) and controls (C). The perfusion pressure and gas transport ability did not differ significantly among the groups. In lungs retrieved from non-heart-beating donors hypercapnia has protective effect against free radical damage caused by room air ventilation during warm ischemia.


The study was supported by the Czech Science Foundation, GACR P303/10/P343, GACR 305/08/0108 and MSMT 1M 0510.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Table 1. Neck circumference and mean pharyngeal area measured in the evening and morning for CHF with OSA, CHF without OSA and healthy controls.

<table>
<thead>
<tr>
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<th>CHF with OSA</th>
<th>CHF without OSA</th>
<th>Healthy Controls</th>
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<tbody>
<tr>
<td>Neck Circumference (cm)</td>
<td>56.3 (54.5-58.1)</td>
<td>58.2 (56.4-60.0)</td>
<td>56.0 (54.3-57.8)</td>
</tr>
<tr>
<td>Mean Pharyngeal Area (cm²)</td>
<td>20.5 (18.9-22.1)</td>
<td>22.0 (20.4-23.6)</td>
<td>20.0 (18.5-21.5)</td>
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*Morning significantly different from evening (p<0.05).


This project was funded by the National Heart and Lung Institute Foundation. It was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London.

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PC284

The asthma associated cytokines IL-13 and TGFβ synergistically increase expression of TRPC6 in human airway smooth muscle

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Asthma has been associated with a defect in intracellular calcium homeostasis of airway smooth muscle (ASM) (Mahn et al., 2010), and we have shown that at least part of this is related to decreased expression of the sarcoendoplasmic reticulum calcium ATPase type 2 isoform (SERCA-2) (Mahn et al., 2009), although increased influx of calcium through channels has also been implicated (Trian et al., 2007). There is little indication that these defects in calcium handling are related to any genetic defect, and evidence from our laboratory and others suggests that at least the reduced expression of SERCA-2 is instead related to the action of asthma-associated inflammatory cytokines such as transforming growth factor β (TGFβ) and interleukin-13 (IL-13). In terms of calcium entry pathways, it has been suggested that channels formed of TRPC6 may make a major contribution to agonist-induced calcium entry in ASM (Mahn et al., 2010). We therefore examined the effects of treatment with TGFβ and IL-13 on human bronchial ASM cells in primary culture, to determine whether these conditions also affected expression of TRPC6. Human ASM cells were cultured from endobronchial biopsies taken from healthy subjects (n=4-6), with informed consent and local ethical permission. Following growth-arrest, cells were stimulated with 10 ng/ml IL-13 and/or 10 ng/ml TGFβ1. TRPC6 mRNA expression was analysed at 6hrs using real-time qPCR and protein expression at 24hrs using Western blot. Antibodies for TRPC6 are notoriously poor, and we therefore first validated a new antibody using a TRPC6-GFP construct expressed in HEK293 cells, using both the TRPC6 antibody (Sigma) and a high quality GFP antibody. In ASM cells, IL-13 had no significant effect on expression of either TRPC6 message or protein. TGFβ1 however significantly increased mRNA expression by a factor of 3 +/- 0.6 (SEM, p<0.05), and in combination with IL-13 there showed a synergistic effect in that expression was increased by a factor of 7.5 +/- 1.1 (P<0.01). Similarly, Western blot revealed no change in TRPC6 protein expression at the predicted MW of ~100kD (114 +/- 14% control, n=4) following treatment with IL-13, whereas TGFβ1 alone caused a significant increase to 181 +/- 22% (P<0.01). Again, TGFβ1 and IL-13 in combination caused a further synergistic increase in TRPC6 protein to 248 +/- 27% of control (P<0.001). These data suggest that the inflammatory milieu associated with asthma causes alterations to both ASM calcium sequestration mechanisms and calcium influx mechanisms, which together could be responsible for the exaggerated response to agonists that is associated with airway hyperresponsiveness in asthma. The apparent synergistic effect of IL-13 and TGFβ1 may be of particular importance, but the underlying mechanisms currently remain obscure.


Asthma UK, MRC, Wellcome Trust

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PC285

The long acting beta agonist formoterol and the cAMP raising agent forskolin both cause reduced expression of SERCA2 in human airway smooth muscle cells

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There is strong evidence that airway smooth muscle (ASM) from asthmatic patients has an altered phenotype, with increased rates of proliferation, migration and secretion of pro-inflammatory cytokines. It has been suggested that a central underlying factor for these changes is a defect in ASM calcium homeostasis (e.g. Mahn et al, 2010). Our laboratory has previously shown that ASM cells from asthmatics exhibit a reduced expression of the sarcoendoplasmic reticulum calcium ATPase (SERCA2), and that knockdown of SERCA2 in healthy ASM cells recapitulates the asthmatic phenotype (Mahn et al, 2009). Whilst the reduced expression of SERCA2 may be primarily associated with inflammatory cytokines, it is unclear what effect current therapies might have. Notably, it has been reported that the long acting beta agonist (LABA) formoterol causes reduced expression of SERCA2 in cardiac myocytes.
Use of burst analysis and agonist concentration jumps to investigate the properties of di- and tri-heteromeric NMDA receptors in dopaminergic neurons of neonatal rat substantia nigra

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While many NMDA receptors are diheteromers composed of two glycine-binding (GluN1) and two glutamate binding (GluN2) subunits, there is evidence for triheteromeric GluN1/GluN2B/GluN2D receptors in the midbrain (Dunah et al., 1998; Jones & Gibb, 2005) and cerebellum (Bickley et al., 2003). Single channel amplitude distributions and ifenprodil inhibition has shown the presence of high conductance GluN2B subunit containing receptors (Jones & Gibb, 2005) while low conductance openings with asymmetrical transition frequencies between high and low conductances suggest GluN2D subunits are present. Here we further utilise ifenprodil block as a tool in steady-state recordings and in agonist concentration jump experiments to test for triheteromeric GluN1/GluN2B/GluN2D receptors in substantia nigra dopaminergic neurons in 300 μm thick midbrain slices from 7 day old rats.

In order to examine the properties of single receptor activations, outside-out patch recordings (n=5) were made in very low concentrations (20nM) of NMDA plus 10μM glycine. Burst length distributions showed that 1μM ifenprodil produced a briefer intermediate burst component (1.43±0.09ms) compared with control (2.56±0.26ms), but no significant change in overall mean burst length (control, 6.67±1.1ms; ifenprodil, 6.63±1.17ms; P=0.9751) or in the slowest component of the burst length distribution (control, 20.44±1.82ms; ifenprodil, 23.56±3.97ms; P=0.3353) that is likely to dominate the response to an agonist concentration jump (Wyllie et al., 1998).

Analysis of the total open time per burst showed a significant decrease in mean open time per burst (control, 2.26±0.38ms; ifenprodil, 1.45±0.21ms; P=0.0228). The time course of the macroscopic response to a brief 4 ms pulse of 1mM NMDA could be best fitted with three exponential components with mean rise-time of 5.49±0.15ms and decay of 59.40±0.41ms (relative amplitude, 10.06±0.10pA) and 1016.0±16.91 ms (relative amplitude, 0.21±0.01pA) (n=5). 1μM ifenprodil inhibited the peak current by 60.4±9.3% (n=5) and decreased the slow time constant to 309.32±10.93ms (relative amplitude, 0.43±0.02pA), but neither the time constant describing the rising (6.03±0.24ms) nor the fast decay time constant (56.48±0.39ms) changed.

These results suggest NMDA receptor activation in substantia nigra dopaminergic neurons produces bursts of channel openings, which combined with the first latencies to activation, generate the familiar slowly rising and decaying macroscopic NMDA response. The co-localization of kinetically distinct GluN2B and GluN2D in a single triheteromeric GluN1/GluN2B/GluN2D receptor may account for the effect of ifenprodil on bursts of openings and on the macroscopic NMDA current decay.


Supported by Henry Lester Trust, G8CET Chinese Student Award and a UCL ORS award

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PC287

Functional characterisation of mutations in the TRESK K+ channel (KCNK18) associated with common migraine


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An inherited mutation in the KCNK18 gene has been shown to be associated with ‘migraine with aura’. This is a common, debilitating, recurrent headache disorder associated with tran-
sient and reversible focal neurological symptoms. KNCK18 encodes the TWIK-related spinal cord potassium channel (TRESK), a member of the K2P family of potassium channels. In a previous study we have shown that a mutation in KCNK18 (F139WfsX24) segregates perfectly with typical migraine with aura in a large pedigree and functional characterisation of this mutation by expression in Xenopus oocytes demonstrates that it causes a complete loss of TRESK function (1). This identifies a role for TRESK in the pathogenesis of typical migraine with aura and further supports the role of this channel as a potential therapeutic target.

In this study we have examined the functional properties of other mutations identified in the human KCNK18 gene and find that whilst some have no obvious effect on channel activity, several variants produce a dramatic loss-of-function phenotype similar to the F139WfsX24 mutation. These results have a major impact on our understanding of how defective TRESK activity may contribute to the pathogenesis of common migraine and the role of common genetic variants in this process.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Impact of ischemic preconditioning on excitatory postsynaptic current (EPSC) and AMPA / kainate-activated currents in primary cortical neurons

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Ischemic preconditioning is described as non-injurious ischemic stimuli that activate different signaling pathways to protect neuronal cells from a subsequent injurious ischemic insult. Rapid ischemic tolerance occurs 30 - 60 min following the preconditioning event, and our recent studies suggest a synaptic mechanism may mediate the protection. Here we report our observations on primary cortical neurons from rat (Sprague-Dawley: 14 DIV) subjected to brief oxygen-glucose deprivation (30 min: ischemic preconditioning). Preconditioning significantly reduced the frequency of EPSCs recorded from cortical neurons, when measured approximately 30-45 min after 30min preconditioning (oxygen-glucose deprivation). Ischemic preconditioning reduced AMPA and kainate-activated current amplitude and increased the desensitization time constant of AMPA-activated current. Concentration response demonstrated a shift to the right in both AMPA and kainate current amplitude. Hence, here we demonstrate for the first time that ischemic preconditioning can regulate EPSC and kinetics of AMPA / Kainate activated currents in neurons to potentially minimize the susceptibility of these cells to ischemic injury.

This work is supported by NIH grant R01 NS059588 to Rob Meller.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Characterisation and pharmacological activation by bithionol of human SLO2.2A K⁺ channels

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Slo2.2 (Slack) channels are structurally related to BKCa channels (Slo1), but rather than synergistic activation by Ca²⁺ and voltage they are activated by intracellular Na⁺ and Cl⁻. Functionally this class of channel (K₁₉₆) is believed to have a role in the control of neuronal excitability and spike frequency adaptation, where channel activity is coupled to Na⁺ during action potentials (1) or excitatory postsynaptic potentials (2). However, the composition of the channels conducting the physiological current has yet to be elucidated. This has been complicated by the identification, in rat, of several N-terminal splice variants, including Slo2.2a (Slack-A), Slo2.2b (Slack-B), and Slo2.2m (Slack-M), in addition to the Slo2.1 (Slick) subunit, which also forms K₁₉₆ channels (3). Bithionol, a bis-phenol antihelmintic compound, has previously been described to activate rat Slo2.2b (Slack-B) channels expressed in both Xenopus oocytes and human embryonic kidney (HEK293) cells (4), however its effect on the other subtypes has yet to be determined. Using whole cell patch clamp of HEK293 cells expressing the human variant of Slo2.2a we have investigated the properties of this homologue and it’s activation by bithionol. Exogenous hSlo2.2a currents were evoked by dialysis of intracellular solution containing Na⁺ 20 mM and currents reversed at EK (-83mV), indicating high K⁺ selectivity. Unlike rat channels, hSlo2.2a did not exhibit time-dependent activation with depolarising voltage pulses and exhibited a linear current-voltage relationship. Bithionol activated hSlo2.2a channels with an EC₅₀ of 1.29 ± 0.12 μM (n=5). Secondly by variance of intracellular Na⁺, between 0-20 mM, in both high (130-150 mM) and low (1mM) [Cl⁻], we found that bithionol activation of hSlo2.2a was not additive but cooperative. With all intracellular Na⁺ concentrations the reversal potential of the bithionol activated current did not significantly deviate from EK (-83mV) (p>0.05, ANOVA). These findings show that human Slo2.2a are voltage-independent K⁺-selective channels that are likely to contribute to the background, rather than delayed-rectifier, K⁺ conductance. Furthermore, Na⁺ and bithionol activate the channels via different modulatory sites. Bithionol could potentially be used to lower excitability in Slo2.2a-expressing cells and would be particularly effective in highly active neurons where Na⁺ influx is frequent.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Effects of hyperthermia on neurogenesis and neurobehavior in the young male rat

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Hyperthermia may cause pathological changes in the brain including, neuronal cell death. In this study, we investigated effects of exogenously induced hyperthermia on neuronal survival, neurogenesis and sensorymotor behavior in young male Sprague-Dawley rats. Three groups of 13-day old rats were used. Body temperature was increased to 39°C (Group I) and 41°C (Group II) in a hyperthermia induction chamber for 30 min. An additional group of animals were used as sham control (Group III; 36°C). Room temperature of the laboratory was 21°C. Core temperature of the animals was monitored by using a rectal probe throughout the experiments. All animals were decapitated 48 h after induction of hyperthermia (day 15). Brains were dissected out and frozen on dry ice. Coronal brain slices (18 μm) were prepared and processed for immunohistochemistry and Cresyl violet staining. To assess the activity, anxiety, and forepaw grip strength of the animals, the open field test, elevated-o-maze, and grip strength test, respectively, were used in all groups 24h before (day 12) and after (day 14) hyperthermia induction. 24 hours before completing the experiments, 100mg/kg (i.p.) BrdU was injected for the evaluation of neuronal proliferation. Experiments were approved by the local ethics committee. Results were statistically analyzed by using One-Way Analysis of Variance followed by LSD test. One day after induction of hyperthermia, animal activity was significantly reduced in both hyperthermia induced groups (p<0.01). Anxiety and grip strength deficits were observed in both test groups; however, it was significant in the 41°C hyperthermia group as compared with control animals (p<0.05). For the evaluation of neuronal survival and neurogenesis, Cresyl violet positive surviving neurons, doublecortin positive neuronal progenitor cells and BrdU positive proliferating cells were evaluated in the cerebral cortex, caudate putamen and hippocampal brain regions.

In conclusion, heat stress induced hyperthermia appears to cause neurobehavioral deficits in post-natal developing male rats. Correlation of these neurological changes with cell proliferation and neuronal cell death in the cortex, caudate putamen and hippocampus with respect to the degree of hyperthermia will be elucidated and reported.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Effects of prenatal hypoxia-ischemic in the motor behavior and cardiovascular system

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Prenatal hypoxia-ischemia (HI) is one of the major causes of mortality and chronic neurological diseases in newborns that can evoke permanent effects such as mental retardation, learning difficulty, epilepsy and cerebral palsy (1). Moreover, changes in fetal development may program the cardiovascular system and lead to an increased risk of cardiovascular diseases. However, the exact mechanisms underlying this relationship remain unknown. Nitric oxide (NO) is a lipophilic gas which has many physiological functions, including vasodilation, neurotransmission and platelet function inhibition. NO bioavailability depends on both its synthesis and its degradation by oxidative stress (2). The aim of our study is to investigate the effects of prenatal HI on oxidative stress, nitric oxide production, and motor and behavior responses in adult rats. HI was induced by clamping the uterine arteries of pregnant rats for 45 minutes, previously anesthetized with trichloroacetic acid injected intraperitoneally (300mg/kg), on the 18th day of gestation (HI group) (3). In the other group of females the surgery was the same, but without clamping the arteries (SHAM group - SH). The weight of the male pups was measured weekly until 90 days postnatal (P90). They were measured for motricity and anxiety by the open field test (OFT) and the plus maze, respectively. The animals in P90 were anesthetized with thiopental and blood collected from the abdominal aorta. Anti-oxidant enzyme catalase activity, thiobarbituric acid reactive substances-TBARS and nitrite (Griess reagent) formation in the serum were measured. The experimental procedures were approved by the Institutional Animal Care and Use Committee (CEA/051/2009) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Data were compared with a one-way ANOVA, expressed by mean±SEM, and significance level was set at 5%, SH n=5 and HI n=10. The HI animals showed reduced weight in comparison with the SH (HI=34,19 vs SH=62,34) and did not show increased anxiety compared with the SH group. The oxidative stress marker, evaluated through TBARS formation (nmol TBA/mg proteins), and anti-oxidant activity of catalase (U/mg of protein) were not modified systemically by HI (TBARS: HI=0.04 vs SH=0.03 and catalase: HI=0.0191 vs SH=0.0122). In relation to nitrite production (mMol/ mg of protein), no significant differences were shown between the HI and sham groups (HI=0.407 vs SH=0.427). Our findings showed that HI impairs motor activity, not affect anxiety and did not cause oxidative stress or affect systemic nitric oxide. Future studies are needed to investigate the relationship of prenatal HI and cardiovascular risk in adults.


This work was supported by FAPERJ and CNPq grants.
PC292

Measuring the dynamics of retino-collicular map formation in the mouse
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Development of retinotopy in the superior colliculus is an area of longstanding investigation. Most studies, however, have yielded mainly qualitative descriptions of the temporal sequences of the topographic development. Here we provide a quantitative description of the temporal sequence of early postnatal topographic development in the superior colliculus of wild-type (C57BL/6) and genetically manipulated mice to inform modelling studies of map formation. The study has been done mainly by utilizing retrograde transport of fluorescent microspheres administered by discrete injections into the superior colliculus in the neonate. All neonatal (P0-P7) animals were anaesthetised using intraperitoneal injection of Ketamine (100-130mg/kg) and Xylazine (10-13mg/kg). Older animals were anaesthetised using inhalation of isoflurane (2%; 0.4-0.6l/min). After fixation perfusion under terminal anaesthesia (300mg/kg pentobarbital sodium), the retinae were removed, flat-mounted and the distributions of labelled ganglion cells plotted. As one measure of order, we take the collicular separation of paired tracer injections that result in separate retinal foci. We find that there are distinct phases of early postnatal refinement. At birth there is only order along the mediolateral (ML) collicular axis. Evidence of order in the anteroposterior (AP) collicular axis is first seen at P2. By P8 ordering in the retinal input has a precision of 315±13μm at P8. Precision refines gradually to reach an adult value of 120±16μm. Using mice lacking the beta2 subunit of the nicotinic acetylcholine receptor (Picciotto et al. 1995), we provide further evidence for a brief activity-dependent critical period for refinement between P4 and P8, showing that precision at P4 is of a similar magnitude as the wild-type, whereas the projection at P8 is significantly less ordered in nAChRβ2−/− animals. While there is some subsequent refinement, the input remains significantly less ordered into adulthood. Picciotto MR, Zoli M, Léna C, Bessis A, Lallemand Y, Le Novère N, Vincent P, Pich EM, Brület P, Changeux JP (1995) Nature. 374(6517):65-7.

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PC293

Oxytocin Alters Spike Activity in Oxytocin Neurons
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The neuropeptide oxytocin is not only an important reproductive hormone, but also regulates emotional and social behaviours such as anxiety and maternal bonding (see eg Broad, Curley and Keverne, 2006). To exert such effects, oxytocin must have an effect on neural behaviour. Earlier studies showed that oxytocin affected the firing frequency of oxytocin neurones in vitro (Inenaga and Yamashita, 1986) and in vivo (Jiang and Wakerley, 1995) but these studies reported increased frequency only and did not show whether it changed the information coded by the spike trains. The present investigation was based on recordings published by (Jiang and Wakerley, 1995). Its aim was to quantify, using the methods outlined by Bhumbra and Dyball (2004) and Sabatier, Brown, Ludwik and Leng (2004), any changes in the patterns of spike activity of oxytocin neurones within the SON that were induced by oxytocin (2.2 ng) injected ICV. There were significant differences in both the mean spike frequency and in the entropy of the log interspike intervals between recordings made before and after administration of oxytocin ICV (paired Student’s t-test, n = 14, P<0.001). Using Pearson’s Correlation Coefficient to assess the relationship between adjacent interspike intervals within the bursts we also found that ICV oxytocin administration significantly increased the complexity of spike coding within the bursts (paired Student’s t-test, n = 29, P<0.01). Both these effects may have been due, in part, to a depolarising action of oxytocin on the OT neurones themselves. They may also reflect more complex actions on other brain regions that modulate the inputs to the SON. Our results do however show clearly that oxytocin, acting centrally, can alter the coding capacity of neurones. It seems likely that such actions of oxytocin, operating at different sites within the brain, may contribute to the mechanism by which oxytocin modulates more complex behavioural patterns.

Acknowledgements: We thank Dr Gary Bhumbra for advice and Dr JB Wakerley for permission to use the relevant digital files.


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PC294

Nitric Oxide (NO) Modulates the Excitability of Magnocellular Neurons of the Supraoptic Nucleus (SON) during changes in Plasma Osmolarity
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Magnocellular neurons of the supraoptic nucleus are responsible for the synthesis of both vasopressin (VP) and oxytocin (OT). Several studies have shown that nitric NO plays a neuromodulatory role on the excitability of these neurons, leading to inhibition of OT and VP release. This effect is related to
increases in plasma osmolality, which also leads to an augmented expression in nitric oxide synthase. In this study, we investigated the role of this nitricergic modulation on the electrical properties of magnocellular neurons subjected to a hypertonic stimulus. We used the patch clamp technique to evaluate action potentials and ionic currents in coronal brain slices of Wistar rats containing the SON. Recordings were made in a control (C) situation (Artificial Cerebro Spinal Fluid (ACSF), 300 mosm/kg/H2O), followed by a hypertonic stimulus (H) (ACSF plus 30 mM manitol, 330 mosm/kg/H2O) and hypertonic stimulus plus 500 microM L-Arginine (H+LA) or 100 microM L-NAME (H+LN). Hypertonicity induces an increase in the firing frequency of the neurons which can be reversed by L-Arginine (C=3.4±0.7 Hz; H=4.9±0.6 Hz and H+LA=4.1±0.5 Hz; n=7, P<0.05). As expected L-NAME produced an additional increase in frequency (C=2.1±0.6 Hz; H=3.1±0.5 Hz and H+LN=3.5±0.6 Hz; n=7, P<0.05). L-Arginine also hyperpolarized the neurons (C=57.3±2.7, H=56.8±2.6 and H+LA=61.2±2.9 mV; n=6, P<0.05) and this effect was reversed by L-NAME (C=-60.3±1.8, H=56.9±2 and H+LN=58.3±2.1 mV; n=5, P<0.05). Changes in membrane potential were also observed in the Hyperpolarization After Potential (HAP), were H+LA induced an increase in its peak (C=74.1±1.7, H=70.9±1.9 and H+LA=73±1.7 mV; n=6, P<0.05). L-NAME produced the opposite response (C=72.9±3.1, H=71.1±3.6 and H+LN=68.5±4.1 mV; n=6, P<0.05). We further investigated whether these alterations were due to nitricergic effects on hyperpolarization activated cation currents (Ih). Our results showed that L-Arginine significantly decreased the steady state values of Ih (measured at -120 mV) in relation to control (C=24.87±1.38; LA=12.39±3.87 pA; n=6, P<0.05). L-NAME induced an increase in Ih (C=12.39±3.87; LN=26.59±9.47 pA; n=6, P<0.05) as well as hypertonicity alone (C=24.11±10.34 vs H=74.23±13.58 pA). Curiously, 30 microM ODQ (a blocker of cGMP) increases Ih (from -18.7±6.7 to -44±4.6 pA; n=4, P<0.05) and ODQ plus L-Arginine decreased Ih to -15.4±3.7 pA (n=4, P<0.05). In conclusion, our results showed that the information about neural decision processes: models can be tested rigorously by their ability to predict latency distributions. In our experiment, whose procedures had local ethical committee approval, we first asked 5 voluntarily-consenting subjects to make saccades to visual targets appearing unexpectedly 3 deg randomly to left or right of a central fixation target. From the distribution of these pro-saccadic reaction times, we estimated best fit values of the LATER parameters μ and α of the basic decision mechanism. They then performed an anti-saccadic task, making saccades in the opposite direction to the targets presented. We tested the ability of a simple competition model to predict the observed latency distributions of both correct (anti-saccade) responses, and those incorrectly made to the target. The model involves a linear race to threshold between units representing antisaccades, pro-saccades, and ‘stop’ (suppression of pro-saccades); there is lateral inhibition between the antisaccade and pro-saccade units, and the starting level of the pro-saccade unit was equal to or lower than that for the antisaccade unit. The values of μ and α for the antisaccade unit and pro-saccade unit were taken to be those from each subject’s control, pro-saccade data. The stop unit α was constrained to be the same for all subjects; stop unit μ, starting level activity of the pro-saccade unit, and the level of lateral inhibition were fitted individually for each subject. A Monte Carlo method was used to simulate distributions, the optimum parameter values being estimated by iterative minimisation of the Kolmogorov-Smirnov statistic. Predicted correct and error response distributions did not differ significantly (p > 0.05) from observed for any of the five subjects. Thus a simple race model can predict the distributions of both correct and error responses in the antisaccade task. As well as advancing our understanding of decision mechanisms, by quantifying clinical antisaccade tests in terms of the underlying functional parameters this can aid the diagnosis and understanding of pathological conditions.

FAPESP and CAPES

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The Timing of Antisaccades

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Antisaccades, saccades made deliberately in the opposite direction to a stimulus, are widely used as a research tool for investigating volitional control as well as neurological and psychiatric disorders [1–3]. Though commonly believed to result from competition in the brain [4] between processes representing normal pro-saccades and antisaccades, we lack both a precise quantitative model that could predict behaviour in detail, and quantitative data by which models could be evaluated.

Response time, or latency, is increasingly used to provide information about neural decision processes: models can be tested rigorously by their ability to predict latency distributions. In our experiment, whose procedures had local ethical committee approval, we first asked 5 voluntarily-consenting subjects to make saccades to visual targets appearing unexpectedly 3 deg randomly to left or right of a central fixation target. From the distribution of these pro-saccadic reaction times, we estimated best fit values of the LATER parameters μ and α of the basic decision mechanism. They then performed an anti-saccadic task, making saccades in the opposite direction to the targets presented. We tested the ability of a simple competition model to predict the observed latency distributions of both correct (anti-saccade) responses, and those incorrectly made to the target. The model involves a linear race to threshold between units representing antisaccades, pro-saccades, and ‘stop’ (suppression of pro-saccades); there is lateral inhibition between the antisaccade and pro-saccade units, and the starting level of the pro-saccade unit was equal to or lower than that for the antisaccade unit. The values of μ and α for the antisaccade unit and pro-saccade unit were taken to be those from each subject’s control, pro-saccade data. The stop unit α was constrained to be the same for all subjects; stop unit μ, starting level activity of the pro-saccade unit, and the level of lateral inhibition were fitted individually for each subject. A Monte Carlo method was used to simulate distributions, the optimum parameter values being estimated by iterative minimisation of the Kolmogorov-Smirnov statistic. Predicted correct and error response distributions did not differ significantly (p > 0.05) from observed for any of the five subjects. Thus a simple race model can predict the distributions of both correct and error responses in the antisaccade task. As well as advancing our understanding of decision mechanisms, by quantifying clinical antisaccade tests in terms of the underlying functional parameters this can aid the diagnosis and understanding of pathological conditions.

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PC295
The effect of coil type and navigation on the reliability of transcranial magnetic stimulation

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Transcranial magnetic stimulation (TMS) is a widely used non-invasive technique for assessing neural mechanisms underlying motor control and recovery from neurological dysfunction. Temporal stability of measurements is important since parameters such as resting motor threshold (RMT), motor evoked potential (MEP) amplitude, short latency intracortical inhibition (SICI) and facilitation (ICF) are often assessed at multiple time-points. The aim of this study was to investigate reliability of TMS parameters for three coil systems: hand-held circular and figure-of-eight coils and a novel, low cost navigated figure-of-eight coil.

Stimulus response (SR) curves, SICI and ICF were studied in the right first dorsal interosseous muscle of 10 healthy human adults after obtaining written informed consent. Each coil system was tested twice per subject over three sessions. SR curves were constructed by delivering 10 stimuli at each of 5 stimulation intensities (90, 100, 110, 120 and 130% RMT). MEP sum was determined by summing the average MEP amplitudes obtained from each of the stimulation intensities. SICI and ICF were investigated by delivering a sub-threshold conditioning stimulus 2.5 ms (SICI) or 12.5 ms (ICF) prior to the supra-threshold test stimulus (Kujirai et al., 1993) and expressing the conditioned MEP amplitude as a percentage of the non-conditioned MEP amplitude. Reliability was assessed using Bland and Altman analyses (Bland and Altman, 1986) and intraclass correlation coefficients (ICCs). Differences between coils were assessed using repeated measures analysis of variance (ANOVA).

Cortical excitability, assessed using MEP amplitude at 120% RMT (MEP₁₂₀) and MEP sum, showed moderate to good reliability for the hand-held and navigated figure-of-eight coils (ICCs 0.55-0.81), but poor reliability for the circular coil (ICCs 0.09 & 0.48). Reliability for SICI was good for all coil systems when an outlier was removed (ICCs 0.87-0.93), but poor for ICF (ICCs < 0.3). The circular coil had a higher RMT than the hand-held figure-of-eight coil (paired t-test: p = 0.016) and a higher MEP₁₂₀ than the navigated figure-of-eight coil (paired t-test: p = 0.004).

These results demonstrate that figure-of-eight coils can be used confidently to investigate cortical excitability over time. ICF measurements should be interpreted with caution. The low cost, easy to use navigation device enables tracking of the position of the coil and subject in real time and frees the experimenter without compromising reliability. The results help guide the choice of coil system for longitudinal measurements of motor cortex function.


make ground contact (Clarke & Harris, 2003). The present study investigated whether a similar differential pattern of reflex sensitization by MO could be found in rats, a species more widely used in pain research. Experiments were performed on 74 rats decerebrated under nitrous oxide/isoflurane (2.25-2.8%) anaesthesia. In addition, 32 rats were spinalized at spinal level T9. Reflexes were evoked in the left knee flexor biceps femoris (BF) and TA by electrical stimulation of skin near the toes, whilst responses in the ankle extensor medial gastrocnemius (MG) were evoked by stimulation of skin at the heel. Responses were recorded as compound EMGs which were averaged and integrated by computer. Conditioning stimuli (5 μl 20% mustard oil), separated by intervals of at least 63 mins, were applied to the IL toe tips, metatarsophalangeal joints (MTJ), mid-sole, heel, dorsal MTJ, instep, ankle joint, knee and lateral gastrocnemius (LG) muscle; the contralateral heel and toes; and the snout and tail. No more than 4 stimuli were applied per animal. Statistical significance was assessed using repeated measures ANOVA and durations of change are expressed as a mean value (minimum n=6 ± SEM). In decerebrate spinalized rats, facilitation of all three reflexes was only elicited by MO application to sites in the IL peritalar region i.e. heel, instep, ankle joint and LG; MO-induced inhibition of responses was absent. For example, MO applied to the heel augmented the heel-MG, toes-BF and toes-TA reflexes (all P<0.001) for periods of ≥22 mins. In decerebrate non-spinal rats potentiation of reflex responses was more restricted. Heel-MG responses were sensitized by MO application to the heel (P<0.03) whilst flexor responses were facilitated from more distal plantar sites. For example, MO applied to the MTJ increased toes-BF and toes-TA reflexes (P<0.03) for 31 ± 9 and 25 ± 7 mins respectively. In contrast to spinalized rats, inhibition of reflexes was observed following MO application to the IL hindlimb as well as off-limb sites. The present data confirm that organization of sensitization of hindlimb reflexes is controlled by descending pathways. Their removal leads to a proximal shift in the sensitization fields of flexor reflexes which implies that both descending inhibitory and facilitatory pathways influence the pattern of MO-induced sensitization in the rat.


This work was supported by BBSRC.

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ings, and extending it by LATER analysis of the distributions, and by refinement of the stimuli so as to make them quantifiable in terms of how much information they provided, and also by using a more natural task (reporting the gender of faces).

We recorded the saccadic latencies of 12 volunteers (see [2] for the methods: the procedures had received local ethics committee approval) in response to targets presented randomly at fixed locations to right and left. In some runs, subjects had to respond to features of the target only visible when fixated by pressing a button, in others no response was required. In three different experiments, the tasks were: detecting a small break in a line, replicating the previous study; reporting the gender of a face; detecting the orientation of a high-frequency Gabor stimulus (with the probability of the stimulus occurring being varied).

In each case, latencies were shorter when subjects expected to obtain useful information from the saccade, with intermediate values for intermediate proportions of Gabors: this reduction was associated with a parallel shift of the distributions, corresponding to a change in the mean rate of rise, μ, of the underlying decision signal (Figure 1).

It is clear that information acquisition reduces saccadic latency in a graded manner. The effect on the distributions was however surprising. Given the primacy of expected utility in determining decisions [3], it would be expected that reward would have the same effect (swivel) on distributions as is well known for probability [4]. However, a change in the rate of rise of a decision signal has been observed in neurons in the caudate nucleus of monkeys rewarded for making some saccades and not others [5], which would be in accordance with our findings.

![Figure 1](image_url)

**Figure 1.** Left, average latency reduction across subjects when subjects use information from the target: Trottier and Pratt (appearance and gap tasks), Gabor (appearance and step), and the faces task. Right, reciprobit plot of latency distributions of one subject in the experiment with variable proportions of Gabors, as indicated. The latency is smaller if the probability of Gabors is larger, and intermediate steps produce intermediate latencies in the expected order, with parallel shift.


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limb peripheral nerves of four patients with diffuse upper limb pain.

Four female patients who presented with fibromyalgia or non-specific arm pain (NSAP) without signs of tissue injury or neurological deficit and six healthy control subjects were recruited. Patients were examined prior to imaging for signs of nerve trunk mechanosensitivity. Transverse images of the median and ulnar nerve were obtained at the wrist using T1-weighted and T2-weighted proton density turbo-spin echo sequences. Coronal images of the brachial plexus were also obtained in the neck of three patients and three controls using a T1-weighted and a T2-weighted Short Tau Inversion Recovery sequence with flow suppression. On the T2-weighted sequences, mean grey scale values were determined from regions of interest drawn over the median/ulnar nerves at the proximal carpal tunnel and distal forearm, and the C6-8 roots of the brachial plexus. Signal intensity ratios were calculated from adjacent soft tissue.

In the control group, there was no difference in median or ulnar nerve signal intensity ratio at the proximal carpal tunnel or distal forearm and therefore the data was pooled (median nerve = 1.21 ± 0.07 SEM; ulnar nerve = 1.40 ± 0.11). There was a significant increase in the signal intensity ratio at the proximal carpal tunnel in the patient group compared to controls for both the median (2.26 ± 0.19 SEM) and ulnar nerve (2.78 ± 0.33 SEM; p<0.05 unpaired t test). In the distal forearm, the signal intensity ratio for both the median (1.63 ± 0.36) and ulnar nerve (1.95 ± 0.34 SEM) returned to control levels (p>0.06). Compared to the controls, there was a 55% increase in signal intensity ratio in the brachial plexus on the most symptomatic side in one NSAP patient. Using validated clinical tests (Kleinrensink et al., 2000), all four patients showed signs of nerve trunk mechanosensitivity to pressure and stretch. A possible cause of the observed nerve signal hyper-intensity in the patients is inflammation, although this cannot be confirmed without histological examination. However, MRI and clinical data from this pilot study suggests that the peripheral nervous system may be involved in the pathogenesis of these diffuse chronic pain conditions.


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CCL2 excites a subgroup of inflamed C-fibre axons in the rat

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There is evidence that chemokines may play an important role in inflammatory pain pathways (Oh et al., 2001). In particular, the chemokine CCL2 and its cognate receptor CCR2 have been implicated in the excitation of injured peripheral neurons (White et al. 2005). The aim of this in vivo study was to examine the electrophysiological effects of CCL2 on both untreated and inflamed C-fibre neurons and to determine the neuronal expression of CCR2 following inflammation.

The sciatric nerve was locally inflamed (neuritis) in anaesthetised adult male Sprague Dawley rats (n=31; isoflurane, 1.75% in O2) as previously described (Dilley & Bove, 2008). Three to six days following neuritis (n=26), and in untreated animals (n=32), dorsal root single unit recordings were carried out under terminal anaesthesia (1.5g/kg urethane i.p.). CCL2 (in 0.1% bovine serum albumin/saline; BSA saline) or BSA saline were suspended for 15 minutes around the sciatric nerve at the neuritis or equivalent site in untreated animals. Axons were assessed for ongoing activity (OA) as a measure of excitability. CCR2 immunofluorescence was carried out on ipsilateral L5 dorsal root ganglia (DRG).

OA developed in 38% of neurons following neuritis (median rate=16.8 spikes/min) compared to 7% in the untreated group (p<0.05). There was also a significant slowing of conduction velocity (median=0.71 m/sec (IQR 0.33)) compared to the untreated group (0.89 m/sec (IQR 0.28); p<0.05). The test agents had negligible effects on untreated neurons (1/10 developed OA post-CCL2, 0/12 post-BSA saline), whereas CCL2 caused 27% (6/22) of neurons in the neuritis group to develop or increase their rate of OA compared to none (0/21) for BSA saline (p<0.05). The ongoing responders had significantly slower baseline firing rates (1.7 (IQR 2.7) spikes/min) compared to non-responders (16.3 (IQR 36.0) spikes/min; p<0.05) as well as normal conduction velocities (0.94 (IQR 0.49 m/sec)). In a subgroup of untreated animals (n=26), the periphery underwent repeated noxious mechanical stimulation during receptive field searches. Receptive field testing took place during in vivo electrophysiological procedures, under terminal anaesthesia. In this group, neurons developed slow OA over time (median rate=2.8 spikes/min). Twenty seven percent (6/22) of these neurons responded to CCL2 treatment compared to 0/19 for BSA saline (p<0.05). The majority of responders (5/6) were recorded late in the experiments (>151 min from setup) and were probably sensitised. CCR2 was expressed at extremely low levels within L5 DRG cell bodies in both the untreated (2.1%) and neuritis groups (0.6%).

In summary, CCL2 excites a subgroup of subtly inflamed C-fibre neurons that are either silent or slowly ongoing and have unaltered conduction velocities. The lack of CCR2 expression on inflamed axons suggests that this receptor is not involved in a direct axonal mechanism in the neuritis model. Since CCL2 effects were rapid, other axonal CCL2 receptors may be involved.

White et al. (2005), Proc Natl Acad Sci USA 102: 14092-14097.

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The gap junction opener trimethylamine enhances characteristics of network based activity within nociceptive dorsal horn

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Neuronal networks in the spinal dorsal horn (DH) present the first site for central integration of peripheral somatosensory inputs. The organization of such networks is not fully understood. In substantia gelatinosa (SG), 4-aminopyridine (4-AP) induces network behaviour that is disrupted by gap junction

Poster Communications

PC302

PC303
Selective decline in slowly adapting type I mechanoreceptors during development in rat sinus hair follicles in vitro

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Studies in primates, including humans, indicate that slowly adapting type I (SA I) mechanoreceptors are responsible for high resolution pattern discrimination by the fingertips (Johnson, 2001). Peripheral terminations of SA I afferents arborize in the epidermis giving rise to disclike endings apposed to Merkel cells. In rodents, the highest concentration of Merkel nerve endings is found in sinus hairs (Halata et al., 2003). Proliferating sinus hairs, present in many animals, are essential tactile organs used to sense the immediate environment: for hunting, food foraging and for spatial navigation. Behavioural studies have shown that the rat’s vibrissa system has a discriminative ability comparable to primate fingertips (Carvell & Simons, 1990). Like the role of SA I mechanoreceptors in primate fingertips, St I mechanoreceptors in the sinus hair follicle complex are likely to play a major role in the highly developed discriminative abilities of rodents and other whisker-bearing animals. It is therefore of considerable interest that of all the sensory nerve endings studied, Merkel endings at the level of the ring sinus (supplied by the deep vibrissal nerve) show the greatest decline and signs of degeneration from 6 weeks to aged (120 week old) rats (Fundin et al., 1997). During our experiments, carried out over the last few years using 6–50 week old rats kept in conventional cages, we found a dramatic drop in the probability of recording from St I units from the deep vibrissal nerve, see Figure 1). A precipitous decline occurred between 6–14 weeks, stabilizing thereafter (although further losses might be expected after 50 weeks during senescence). In contrast, the prevalence of St II units remained fairly constant over the age range. The decline in prevalence of St I units that we see from 6–14 weeks corresponds to the reduction in Merkel nerve endings seen in other studies from 6–120 weeks (Fundin et al., 1997). We hypothesise that there may be two components: an early decline from young adulthood due to a pruning of underused St I mechanoreceptors, and a much later decline only observed after 1–2.3 years associated with senescence. The decline in Merkel nerve endings may be linked with a reduction in neurotrophin 3 and trkC (reviewed by (Ulfhake et al., 2002)), and a loss of Merkel cells rather than the myelinated nerves that supply them, and as such it may be possible to preserve or restore peripheral cells (Merkel cells) as targets for therapy.

Figure 1. A plot of mean number of St I and St II units plotted according to the age of rat. ANOVA comparing the incidence of the two types of units from 112 experiments (112 different animals) over the last few years showed there was a statistically significant interaction between type of unit and time $(F(8,103) = 2.8, p = .008$. The Figure shows that St I units in 6 week old animals were approximately 2.5 times as common compared with animals older than 14 weeks. Pairwise t test comparisons showed that St I units were significantly more common than St II units at the youngest mean age of 6 weeks ($t(10) = 3.99, p = .003$, while St II units were more common at mean age of 22 weeks ($t(25) = 2.51, p = .02$).– see asterisks on Figure, and St II units tended to be more common from that age up to the maximum age studied of 50 weeks.


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L-Arginine-nitric oxide pathway and possible implications for cardiovascular disease in depressive patients

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Introduction: Major depressive disorder (MDD) represents an important cardiovascular risk factor, and patients with this disorder have twice the cardiovascular mortality expected from the general population (1, 2). However, the exact mechanisms underlying this relationship remain unknown. Studies suggest the involvement of the L-arginine-nitric oxide (NO) pathway in the pathogenesis of MDD (3). NO is responsible for several physiological functions, including platelet function inhibition, neurotransmission and vasodilatation (4). The aim of study was to investigate the role L-arginine-nitric oxide pathway in platelets from patients with MDD. Methods: Ten patients with MDD meeting DSM IV criteria and without any medication (4 platelets from patients with MDD. Ten normal control subjects (CS). The Pedro Ernesto Hospital Ethical Committee approved this study (1436– CEP/HUPE), and informed consent was obtained from each participant. Extracellular L-arginine transport into platelets was measured by kinetic methods, using crescent concentrations of [3H] L-arginine. NOS activity was evaluated by the conversion of [H3]L-arginine into [3H]-citrulline, and cGMP content was determined in washed platelets at baseline using a commercial ELISA method. L-arginine concentration was measured by high-performance liquid chromatography (HPLC) method. The Mann-Whitney U test or unpaired test was used to analyze the differences between MD and (CS), in accordance with the Kolmogorov-Smirnov test. Values were expressed as means ±SEM. A p value of less than .05 was considered significant. Results: L-arginine transport via the y+L system (pmol/109 cells/min) into platelets was decreased in MDD (CS: 46±9, MDD: 20±2, p<.05). Basal NOS activity (pmol/108 cells) was diminished in platelets from MDD (0.16±0.01) compared to CS (0.09±0.01), p<.05. cGMP content (pmol/108 cells) was reduced in MDD (0.04±0.01) compared with CS (0.11±0.02), p<.05. L-arginine concentration (µM) was also decreased in plasma from MDD patients (104±4) in relation to CS (130±8), p<.05. Discussion: We have verified an impairment of L-arginine transport into platelets with subsequent reduced NO production cGMP content. A diminished concentration of plasma levels L-arginine could reduce transmembrane transport and consequently intracellular substrate for NOS in platelets with MDD. It is possible that an impairment of the L-arginine-NO-GMPc pathway might be an early marker of future platelet activation and thrombotic events in MDD patients.

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X-ray crystal structure of a prokaryotic inwardly-rectifying (KirBac) potassium channel reveals the mechanism of channel opening at the bundle-crossing gate

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Inwardly-rectifying (Kir) potassium channels are important regulators of cellular electrical activity and excitability and inherited mutations in these channels underlie a wide range of channelopathies. Yet despite the wealth of high-resolution structural information which now exists for this class of K+ channels, the mechanism by which they open and close, and how this process is regulated are still not fully understood. The primary gating mechanism is thought to involve opening and closure of the conductive pathway at the cytoplasmic bundle crossing. However, all of the available crystal structures of either eukaryotic Kir or prokaryotic KirBac channels are fully closed at the bundle crossing gate and there are currently no available structures of a Kir channel with the bundle-crossing in an open conformation.

In a previous genetic complementation study we identified a range of gating mutations in a prokaryotic KirBac channel and we have now used these mutants to help determine the X-ray crystal structure of a KirBac channel at 3.05Å resolution. This novel structure reveals a channel with the transmembrane helices in an open conformation as well as highlighting a number of important interactions between the cytoplasmic assembly and the transmembrane helices that are likely to be essential for maintaining the channel in the open state. This structure therefore provides an important structural insight into the mechanism of Kir channel gating and our understanding of how this process can be regulated by physiological ligands.

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Functionality regulation of transient receptor potential vanilloid type(TRPV) 6 channel by extracellular pH

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The major role of TRPV6 has been known to absorb calcium in small intestine from the food. The amino acid sequences of TRPV6 have 30% homology with TRPV5, but the homology in pore helix is about 75% and their structures in pore helix are very similar. The extracellular pH sensitivity of TRPV5 have studied already, but TRPV6 has not done yet. Maybe this is because the organs where TRPV6 distributes are generally insensitive to extracel-
lular pH, and therefore, it’s difficult to determine physiological meaning of extracellular pH sensitivity of TRPV6. As it is known that calcium is very important for wound healing and aging in skin, there is a possibility TRPV6 play an important role in the calcium concentration regulation in epidermis. It is known that epidermal and dermal pHs are different each other and their calcium concentrations are easily variable. Therefore, this study was planned to check extracellular pH sensitivities of TRPV6 which is related with wound healing and/or skin aging.

For this, extracellular pH sensitivity of TRPV6 was examined using whole-cell patch clamp technique in CHO(chinese hamster ovary) cells. The pKa, 50% of currents is inhibited from the maximum, was 5.71±0.27. The 521st histidine was insensitive to extracellular pH changes, so it may play as an extracellular pH sensor.

To explain the mechanism how proton affects the function of TRPV6 substituted cysteine accessibility method(SCAM) was performed. The currents of 521st histidine to cysteine mutant is possibly inhibited by MTSET and the mechanism for such a phenomenon is possibly due to conformational changes.

In conclusion, the 521st histidine plays a role as extracellular pH sensor and it causes the functional declines not by direct blocking effect or electrostatic effect but by conformational changes through proton binding. The pKa values of a variety of TRPV6 mutants

<table>
<thead>
<tr>
<th>Location of mutated amino acids</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
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<td>Wild type</td>
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<td>4.52</td>
<td>3.99</td>
</tr>
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<td>7.31</td>
<td>2.50</td>
<td>4.99</td>
</tr>
</tbody>
</table>

Yeh BI et al. (2005). EMBO J, 24(18), 3224-3234.
Hoenderop JG et al. (2003), EMBO J 22, 1-10.

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PC308

Protein Kinase A (PKA) is central for the forward transport of two-pore domain K⁺ channels K₂P3.1 and K₂P9.1

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Acid sensitive two-pore domain K⁺ channels (K₂P3.1 and K₂P9.1) play key roles in physiology and disease, the most fundamental of which is control of the resting membrane potential of cells(1). As these ‘leak’ channels are constitutively active once expressed at the plasma membrane, tight control of surface expression is fundamental to the regulation of K⁺ flux and cell excitability. The chaperone protein, 14-3-3, binds to a critical phosphorylated serine in the C-termini of K₂P3.1 and K₂P9.1 (S393 and S373, respectively) and overcomes retention in the endoplasmic reticulum by βCOP (2-4). We sought to identify the kinase responsible for phosphorylation of the terminal serine in human (h) and rat (r) K₂P3.1 and K₂P9.1. We tested the effect of mutating the terminal serine to alanine (S393A) on the function of hK₂P3.1 expressed in Xenopus oocytes by two electrode voltage clamp. This mutation resulted in a total loss of current, as did the double substitution S392A/S393A or the removal of the terminal valine (ΔV394). Three candidate kinases were identified: CAMP-dependent protein kinase (PKA), ribosomal S6 kinase (RSK,) and protein kinase C (PKC). In vitro phosphorylation assays supported in silico predictions: PKA phosphorylated the terminal serine of both hK₂P3.1 and h and rK₂P9.1. RSK2 phosphorylated the terminal serine of hK₂P3.1 effectively in vitro, but PKC did not appreciably phosphorylate any of the C-termini tested. Whole cell patch clamp measurements of hK₂P3.1 expressed in HEK293 show a negative shift in resting membrane potential from -32.6 mV (S.E.M. 1.16, n=6) in non-transfected cells to 54.6 mV (S.E.M. 3.12, n=14) in channel-transfected cells. For cells transfected with hK₂P3.1 and cultured in the presence of the constitutive PKA activator, 8Br-cAMP (0.4 mM), increased current was observed at test potentials between -50 mV and +90 mV. At 60 mV, evoked current increased from 0.59 pA (S.E.M. 0.05, n=7) to 1.09 pA (S.E.M. 0.14, n=9) in 8Br-cAMP treated cells, with a modest negative shift in the resting membrane potential to -58.6 mV (S.E.M. 2.45, n=7). Conversely, in HEK293 cells incubated with two different PKA inhibitors (1 μM KT5720 or 20 μM myristoylated PKA-specific inhibitor), there was a decrease in current at all test potentials when compared with non-treated cells (significant: P<0.05). Unexpectedly, the RSK inhibitor, SL0101, caused an increase in current (1.01 pA, S.E.M. 0.12 at 60 mV, n=8), but without a concomitant decrease in membrane potential compared to non-treated matched controls. Immunofluorescence and flow cytometric measurements of GFP-tagged K₂P3.1 and K₂P9.1 expressed in HEK293 cells supported the in vitro phosphorylation and electrophysiology conclusions: PKA is responsible for the phosphorylation of the terminal serine in both K₂P3.1 and K₂P9.1 (5).


This work was funded by BBSRC award BB/E014453/2 to IO’K.

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Correction of the Cystic Fibrosis Transmembrane Conductance Regulator in Cystic Fibrosis Epithelial Cells using Zinc Finger Nuclease Homology-Directed Repair

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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a cAMP regulated ion channel, allows Cl- flux across the apical membrane of epithelial cells. Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in CFTR. The most common mutation a CTT deletion (∆F508) disrupts CFTR channel activity. Cl- flux can be restored following the addition of CFTR cDNA to a model CF cell line (Rich et al., 1990). However, attempts to deliver CFTR cDNA to the lungs of patients to restore function have not resulted in any clinical benefit (Davies and Alton, 2010).

An alternative approach is to correct the mutations in the genome. Gene correction involves introduction of a donor DNA molecule containing the correct sequence to cells which then triggers homology-directed repair (HDR) of the mutant gene. Advantages include permanent correct temporal spatial expression and restoration of any splice variants. However, the efficiency of HDR can be as low as 1 in 106 treated cells.

Double stranded breaks (DSBs) in the target gene created by synthetic restriction enzymes zinc finger nucleases (ZFNs) can increase the rate of HDR using a donor sequence to 1 in 5 treated cells. Our results show oscillatory changes of the [Cl-]i in SiHa cells during cell cycle progression in SiHa cells. Cells were transiently transfected with Cl-sensor and then synchronized into different stages of cell cycle. G0 phase cells were prepared by a 72 h cultivation in serum-free medium, G1 phase cells by 5 μM aphidicolin treatment for 24 h, S phase cells were prepared by 5 μM aphidicolin treatment for 24 h followed by a 4 h release in fresh medium, and G2 phase cells by 5 μM aphidicolin treatment for 24 h followed by an 8 hr release in fresh medium. M phase cells were prepared by 10 μg/ml nocodazole treatment for 18 h and thereafter the shake-off cells were collected. Fluorescent images were acquired using an IonOptix system with excitation filter (440/480 nm) and emission filter (above 510 nm). The intensity ratios (F480/F440) were determined. Calibration was via nigericin/tributyltin chloride treatment.

Our results show oscillatory changes of the [Cl-]i in SiHa cells as the cell cycle progresses. [Cl-]i rises sharply (from 10 to 40 mM) in S phase. This level is maintained in G2 but then decreases markedly to 20 mM in M phase. Similar trends in oscillatory changes of the [Cl-]i were also found in KCC3-overexpressed cells and ∆N117 mutant cells. [Cl-]i rises from 5 to 20 mM (KCC3-overexpressed cells) or 15 to 60 mM (∆N117 mutant cells) in S and G2 phase and then decreases to 15 mM (KCC3-overexpressed cells) and 40 mM (∆N117 mutant cells) in M phase (Fig.1). The present results not only imply that the regulation of intracellular chloride concentration is an important factor in controlling cell cycle progression but also provide a clue for a critical regulatory role for cation chloride cotransporters in the aetiology of epithelial cancers.
Figure 1. Changes of intracellular chloride concentration during cell cycle progression in SiHa, KCC3, and ΔN117 cells


FAESP

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PC311

P2X subunits expression in mouse Leydig cells at different ages

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ATP acts on the plasma membrane by interacting with purinergic receptors P2X (ligand-gated ion channels) and P2Y (G-protein-coupled receptors). Until now, seven subunits of P2X receptors were identified and cloned (P2X1 - P2X7). They form functional receptors with distinct biophysical and pharmacological properties depending of their structure. Calcium influx through these channels is in part responsible for the increase in intracellular [Ca2+] observed in a number of physiological situations. ATP treatment of Leydig cells from mice and rats, leads to an increase in [Ca2+] and testosterone secretion. As testosterone production is an age dependent process, our hypothesis here is that the pattern of specific P2X subunits expression in Leydig cells changes along the development. Based on the developmental stages defined by Wu et al. (2010) the purpose of this work was to investigate the expression of P2X receptor subunits in mouse Leydig cells aged 07, 14, 21, 24, 28, 35, 45, 60, 75 and 90 days old. Western blot experiments were performed in freshly isolated Leydig cells purified in a percoll discontinuous gradient essentially as described in Salva et al., (2001). Polyclonal antibodies (Alomone Labs Ltd., Jerusalem, Israel) (1:200) against P2X2, P2X4 and P2X7 subunits were used in order to detect a particular type of receptor. Our results showed P2X2 expression from day 28 till day 100, P2X4 expression from day 21 till day 100 and P2X7 expression from day 35 till day 75. The expression pattern of P2X4 and P2X7 subunits matches the temporal expression of the steroidogenic enzymes P450scc and P450c17alpha (day 24 till day 90) and the expression pattern of P2X2 match the plasma testosterone levels in mice (day 24 till day 180) as describe by Wu et al., 2010. We suggest that purinergic P2X receptors in mouse Leydig cells could modulate the steroidogenic process and influence testosterone production.

Supported by the M.R.C. Grant G0700759

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PC312

Epidermal growth factor-driven expression of a TTX-sensitive voltage-gated sodium channel potentiates human non-small cell lung cancer invasion via elevation of intracellular sodium concentration

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Expression of NaV channels has been reported in a range of cancer cells and appears to be specifically linked to cell invasion. However, the cellular mechanisms underlying how NaV channels promote invasion remain unclear. We have shown previously that EGF receptor stimulation enhances H460 NSCLC invasion via expression of a TTX-sensitive NaV1.7. We are currently attempting to define the interplay between intracellular sodium and metastatic potential in invasive NSCLC cell lines. The presence of NaV channel currents in strongly metastatic (H460) and weakly metastatic (A549) NSCLC cell lines was determined using patch clamp electrophysiology with physiological saline solutions. ICC was carried out on formaldehyde-fixed cells permeabilised with saponin. Gene expression was assessed by qPCR using cDNA obtained from 25ng total cell RNA, with all results normalised to 18S rRNA expression. Protein levels were determined with western blots using 20ug total cell protein. Intracellular Na+ levels were measured using the ratiometric dye SBFI. Cell invasion was analysed using a Boyden chamber setup with Matrigel™. Values are means ± SEM, compared by unpaired Student’s t-test or ANOVA. EGF-regulated, TTX-sensitive NaV1.7 currents are present in H460 cells but absent in A549 cells. Moreover, the current in the H460 cells is a sustained one at the resting membrane potential of the cells (-27±4mV, n=6). H460 cells have an internal Na+ concentration more than double that of the A549 cells (H460: 22.3±4.3mM, n=4, A549: 9.7±3.2mM, n=4, p<0.01) and when NaV1.7 channels are blocked in H460 cells with 1μM TTX, or downregulated with 5nM siRNA directed against NaV1.7, cell invasion is significantly reduced (CTL: relative invasion = 1.00±0.05, n=9, TTX: 0.60±0.07, n=9, siRNA: 0.50±0.02, n=9, p<0.01 and p<0.001 for TTX and siRNA vs. CTL respectively). Additionally, when the sodium/hydrogen exchanger protein NHE1, whose expression is also regulated by EGF signalling, is blocked in H460 cells with EIPA, cell invasion is also...
significant decrease and there is no additive effect following co-application of EIPA and TTX (CTL: relative invasion = 1.00±0.03, n=9, EIPA: 0.60±0.02, n=9, EIPA+TTX: 0.62±0.04, n=9, p=0.001 vs. CTL for both treatments). These data therefore suggest that elevated intracellular Na+ is driving H460 cell invasion. Preliminary work reveals that treatment of H460 cells with 10μM monensin (Na+ ionophore) significantly enhances cell invasion (CTL: relative invasion = 1.00±0.03, n=6, monensin: 1.80±0.19, n=8, p<0.001), thus supporting the idea that elevated intracellular Na+ alone can drive cancer cell invasion. Work is in progress to determine the precise mechanism downstream of Na+ that may be driving cell invasion, with specific focus on Ca2+ signalling and pH.

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PC313

Modulation of TRPM3 cation channel by intracellular Ca2+
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TRPM3 is a non-selective cation channel that is widely expressed throughout the body and it has been implicated in the regulation of vascular smooth muscle cell function (Naylor et al, 2010) and insulin secretion (Wagner et al, 2008). However, its regulation remains incompletely understood. Other members of the melastatin subgroup of TRP channels such as TRPM2, TRPM4b and TRPM5 are strongly activated by intracellular Ca2+. Therefore we tested the hypothesis that TRPM3 is also a Ca2+ activated ion channel. The effect of Ca2+ on TRPM3 function was investigated using a calcium indicator dye (fluor4) and the whole-cell patch-clamp technique. HEK 293 cells stably expressing human TRPM3 were used in these experiments. Data are shown as mean ± s.e.m. TRPM3 expressing cells were treated with thapsigargin to elevate intracellular Ca2+. These cells displayed an increase in basal intracellular calcium levels and their response to PregS was decreased by approximately 20 % (n=3). This result indicates that TRPM3 is inhibited by increases in intracellular Ca2+. Patch-clamp experiments however, revealed that Ca2+ has a more complex effect on TRPM3. Under conditions of low Ca2+ buffering (0.1 mM EGTA in the pipette solution) two effects of Ca2+ were observed. In 4 out of 9 whole-cell patches where the extracellular solution was switched from a Ba2+- to a Ca2+- containing solution the PregS induced inward and outward currents were significantly potentiated (2.9±0.3 and 1.9±0.1 times respectively). In 4 of the remaining patches application of Ca2+ caused a significant inhibition of the inward and outward PregS induced currents (44±4 and 77±7 % respectively). All of these effects were lost under high Ca2+ buffering conditions (10 mM EGTA in the pipette solution) suggesting they arose due to changes in intracellular Ca2+.

The data suggest that TRPM3 is both stimulated and inhibited by intracellular Ca2+. Experiments are ongoing to reveal the underlying mechanisms and physiological relevance.


This work is funded by the British Heart Foundation.

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PC314

Characterisation of a chloride conductance in canine chondrocytes
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Healthy chondrocytes can exist with strikingly depolarised resting membrane potentials (RMP) (Wright et al., 1992; Wright et al., 1996). Several papers have investigated the role of potassium conductances in control of RMP (including Wilson et al., 2004) and we showed that the RMP was also dependent upon TRPV5 (Lewis et al., 2011). In the same study we showed that this depolarised RMP was crucial to the control of chondrocyte volume. A number of studies have also suggested that chloride channels are important for control of the RMP (reviewed by Barrett-Jolley et al., 2010) so in this study we investigated the functional expression of these channels in chondrocytes, using both inside-out patch clamp and whole-cell electrophysiology.

Chondrocytes were isolated from canine articular cartilage by standard methods (Lewis et al., 2011). Cells were used up to and including the third passage. For inside-out patch experiments, membrane potential (Vm) was calculated as Vm = −Hp−Vj where Hp was the holding potential and Vj the calculated junction potential. Data are expressed as mean ± standard error, p-values are from unpaired t-tests.

We identified a population of ion channels with a mean slope/unitary conductance of 183±3pS (n = 5) using inside-out patch experiments. These channels reversed at a membrane potential of −34±6mV (n = 5) in the presence of 40mM internal and 158mM external Cl− solutions, indicative of a chloride current (calculated equilibrium potential, ECl ~ −35mV). This channel activity was inhibited by the chloride channel blocker 4-Acetamido-4’-isothiocyanato-stilbene-2,2’-disulfonic acid (SITS) at a concentration of 100μM and seen in approximately 30% of patches with a mean open probability (Po) of 0.7±0.1 (n = 3). Application of 100μM SITS decreased channel Po by 83±6% (n = 3; p<0.05). In whole-cell voltage clamp mode, 100μM SITS inhibited voltage ramps by 52±6% (n = 4; p<0.05) at 20mV. We investigated the effect of SITS on the RMP with whole cell current-clamp experiments and found 100μM induced a significant change of +12±3mV (n = 5; p<0.01).

SITS is a relatively non-selective inhibitor of anionic currents so, to further characterise this chloride current, we used a more specific channel inhibitor; niflumic acid (NFA). NFA inhibits the calcium-activated chloride channel (CaCC), which is also believed to be a volume-sensitive chloride channel. 100μM NFA inhibited whole-cell current by 18±2% (n = 15; p<0.01) at 20mV. Our combined single channel and whole-cell data are consistent with the expression of a mixed population of chloride channels in chondrocytes, including both high conductance maxi-chloride and CaCC-like channels.


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PC315

Prenatal exposure of pregnant rats to cigarette smoke and nicotine: effect on nitric oxide and fasting glycemia in treated and untreated neonates with vitamin C

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Smoking remains a serious public health problem. It is still unclear whether the injurious effect of cigarette smoking in pregnancy are due to nicotine, and if so, what the effects are. The effects of cigarette smoking on nitric oxide (NO) pathways and nitric oxide synthase (NOS) isoenzymes are controversial and may vary according to the disease, model or location of the NOS. It has been documented that cigarette smoke inhibits NO production in active and passive smokers (1). This study investigated the effects on plasma nitric oxide and fasting glycemia in neonates from pregnant rats exposed to cigarette smoke and nicotine, postnatally treated and untreated with vitamins C. Fifteen female wistar rats weighing 250-300g were divided into three groups of five rats each. Group A rats were exposed to idling cigarette smoke in an ad hoc chamber for 30 min per day from day 0 to day 10 of gestation. Group B received 0.25 mg/kg body weight of nicotine intramuscularly while Group C served as the Control. After mating and gestation, litters from each group were randomly subdivided into two groups of ten neonates each. Group A: A1 and A2, Group B: B1, and B2, Group C: C1 and C2. Groups A1, B1 and C1 received 0.25 mg/kg B.W Vitamin C (50mg/kg body wt administered orally in a suspension) for 4 weeks after birth while Groups A2 and B2 and C2 did not receive any. Plasma nitric oxide and fasting glycemia were estimate in blood samples obtained from these animals (taken from heart puncture after 1.9% ether anaesthesia). Values are means ± S.E.M., compared by ANOVA. Results show that the neonates of animals exposed to cigarette smoke had a significantly higher nitric oxide concentration (21.00 ± 1.29 μM) than those from not exposed ones (10.75 ± 2.95 μM) and a significantly lower fasting glycemia (61.00 ± 2.03 mg/dl) when compared with the control (75.50 ± 3.73 mg/dl). In contrast, prenatal exposure to nicotine neither had a significant effect on nitric oxide concentration (12.50 ± 2.10 μM) nor on fasting glycemia (74.17 ± 3.48 mg/dl) when compared with the control (10.75 ± 2.95 μM) and (75.50 ± 3.73 mg/dl), respectively. While the effects of cigarette smoke shown here could not be attributed to the pharmacological activity of nicotine, they may be related to the formation of smoking induced oxidative free radicals, since the administration of an antioxidant as vitamin C reversed them.


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PC316

Cytokine regulation of natural antimicrobial peptide expression in cultured human vaginal epithelial and endocervical cells


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Introduction: Natural antimicrobial peptides (NAPs) have been identified, in the female reproductive tract (King et al., 2007) and in cervico-vaginal fluid (Stock et al., 2009), as an integral part of innate immune defence. The role of NAPs in spontaneous preterm birth is not well described. Based on data obtained in vivo (Chandiramani et al., 2010), we hypothesised that granulocyte-macrophage colony-stimulating factor (GMCSF) and other pro-inflammatory cytokines (an early indicator of inflammation and risk of preterm birth) would regulate expression of NAPs in reproductive tract epithelia. Our aim was to assess impact of interleukin-1beta (IL-1β) and GMCSF on elafin, secretory leucocyte protease inhibitor (SLPI) and human-beta defensin-2 (HBD-2) mRNA expression in vaginal epithelial (VK2/E6E7) and endocervical (END-1) cells. Methods: VK2/E6E7 (VK2) and END-1 cells (80% confluence) were treated with IL-1β and GMCSF (1 ng/ml and 10 ng/ml) for 6 and 24 h. Elafin, SLPI and HBD-2 mRNA expression, relative to stably expressed GAPDH (VK2 cells) or a normalisation factor of 3 housekeeper genes (END-1 cells), was assessed by quantitative real-time PCR. Data are expressed as mean (±SEM) and analysed using analysis of variance with repeated measures and a Bonferroni’s multiple comparison post-hoc test or Freidman’s test with Dunn’s post-hoc analysis. Experiments were performed in triplicate for n=6 passages for VK2 cells and n=3 for END-1 cells. Results: Elafin, SLPI and HBD-2 transcripts are present in VK2 and END-1 cells. In VK2 cells, IL-1β (10 ng/ml) significantly increased elafin mRNA expression (14.28 ± 4.42 vs control 3.15 ± 1.62, n=6, p<0.001), SLPI (7.91 ± 3.60 vs control 6.80 ± 0.99, n=6, p<0.05) and HBD-2 (0.016 ± 0.0052 vs control 0.001 ± 0.0004, n=6, p<0.001). GMCSF (10 ng/ml) significantly inhibited elafin in VK2 cells (2.53 ± 0.68 vs control 6.43 ± 1.92, n=6, p<0.05). In END-1 cells, IL-1β (1 ng/ml) significantly increased elafin mRNA expression (146260 ± 24314 vs control 77388 ± 22017, n=3, p=0.0001) and HBD-2 (IL-1β 10 ng/ml) (48.47 ± 10.15 vs control 9.06 ± 2.92, n=3, p=0.007). GMCSF (1 ng/ml) significantly inhibited elafin expression in END-1 cells; (19631 ± 4697 vs control, 29896 ± 37300, n=3, p=0.02) and SLPI (10 ng/ml)(26758 ± 3741 vs control 38458 ± 3278, n=3, p=0.01). GMCSF had no effect on HBD-2 expression in either cell line. Conclusions: This study provides valuable information on the regulation of NAPs by inflammatory cytokines in vaginal epithe-
PC317

Mirror-like SeSAME/EAST renal phenotype in mice lacking the Kir5.1 (Kcnj16) K⁺ channel subunit

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The heteromeric inwardly-rectifying Kir4.1/Kir5.1 K⁺ channel underlies the basolateral K⁺ conductance in the distal nephron and is extremely sensitive to inhibition by intracellular pH (pHi). The functional importance of Kir4.1/Kir5.1 in renal ion transport has recently been highlighted by mutations in the human Kir4.1 gene (KCNJ10) which result in SeSAME/EAST syndrome, a complex disorder that includes salt wasting and hypokalaemic alkalosis. Here, we have examined the role of the Kir5.1 subunit, using mice we have previously created that have a targeted disruption of the Kir5.1 gene (Kcnj16). The functional importance of Kir4.1/Kir5.1 in renal ion transport has recently been highlighted by mutations in the human Kir4.1 gene (KCNJ10) which result in SeSAME/EAST syndrome, a complex disorder that includes salt wasting and hypokalaemic alkalosis. Here, we have examined the role of the Kir5.1 subunit, using mice we have previously created that have a targeted disruption of the Kir5.1 gene (Kcnj16) (2). Interestingly, we find that the Kir5.1/-/- mice display a phenotype that is opposite of that seen in SeSAME/EAST syndrome where the Kir4.1 subunit is dysfunctional and leads to an overall down-regulation of Kir4.1/Kir5.1 functional activity. We suggest that this mirror-like phenotype may be a consequence of the fact that the Kir5.1 subunit is present in these mice, and that the remaining homomer Kir4.1 subunits cause an increased K⁺ conductance due to their reduced pH-sensitivity. These results highlight the important role that heteromeric Kir4.1/Kir5.1 plays as a pH-sensitive regulator of salt transport in the distal convoluted tubule. (1) Bockenhauer et al (2009) N Engl J Med. 360:1960-70. (2) D’Adamo et al. (2011) J Biol Chem. 286:192-8.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC318

Regulation of the epithelial sodium channel (ENaC) by norepinephrine in cultured mouse renal collecting duct cells

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Sodium absorption in the aldosterone-sensitive distal nephron (ASDN) plays an important role in the maintenance of body sodium balance and the long term regulation of arterial blood pressure. This absorption is mediated via the epithelial sodium channel (ENaC) which can be regulated by hormones and other factors (1). Increased renal sympathetic nerve activity has been shown to decrease renal Na⁺ excretion. This may be attributed to increased tubular Na⁺ absorption. Recently, a clinical study has demonstrated that renal denervation significantly reduced blood pressure in patients with resistant hypertension [2]. However, the regulatory role of renal sympathetic innervation in tubular Na⁺ transport is still unclear. Therefore, we investigated the effects of norepinephrine on ENaCmediated transepithelial Na⁺ transport in mCCD11c cells, a model of renal collecting duct principal cells. These cells endogenously express ENaC and respond to physiological concentrations of aldosterone and vasopressin [3, 4]. Na⁺ transport across cells cultured on permeable supports was monitored using Ussing chambers to record equivalent short circuit current (Isc). Data are shown as mean ± SEM. Basal Isc averaged 5.1 ± 0.3 μA cm⁻² (n=7) and was inhibited by apical addition of 100 μM amiloride. This indicates that basal Isc can be attributed to electrically driven Na⁺ absorption via ENaC. Basolateral addition of norepinephrine (10 μM) produced a peak increase in Isc that returned to the baseline value within less than one minute. This was followed by a transient inhibition of the Isc by 1.2 ± 0.1 μA cm⁻² (n=7) within ~15 min. Over the subsequent 2.5 h, Isc gradually increased by 2.2 ± 0.5 μA cm⁻² above baseline (n=7, p < 0.01, paired Student’s t-test). In contrast, Isc did not increase in matched vehicle-treated cells (0.2 ± 0.4 μA cm⁻², n=7). The Isc component stimulated by norepinephrine was inhibited by amiloride. Apical application of norepinephrine did not alter Isc. When amiloride application preceded the addition of norepinephrine, only the initial Isc peak remained which may be caused by a transient Cl⁻ secretory response. In the presence of amiloride, norepinephrine failed to elicit the transient inhibition and the sustained increase in Isc. This indicates that the transient inhibitory and the sustained stimulatory response to norepinephrine can be attributed to an inhibition and a stimulation of ENaC activity, respectively.


Funded by the Bayerische Forschungsstiftung.
Quality of life in children with type 1 diabetes in Kuwait

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Introduction: Recent research has shown that health-related quality of life (HRQOL) in children and adolescents with type 1 diabetes is markedly affected, resembling that of children with other chronic diseases, like malignancies. The objective of the study was to investigate the HRQOL in children and adolescents with diabetes in Kuwait. Method: A total of 341 children and adolescents aged 5-18 years and 408 parents of children aged 2-18 years participated in the study. They were recruited from diabetes out-patient clinics in the 6 governorate hospitals. The pediatric quality of life inventory (PedsQL) questionnaire was used. Results: The mean (±SD) age of participants was 9.9±1.2 years, and the duration of diabetes was 4.9±2 years. The Cronbach’s coefficient and child and parent report generally approached 0.825, indicating their internal consistency and reliability. There was a statistically significant difference in the total scores among children and their parents in all 3 age groups (p < 0.001), however, to a lower degree in the adolescent group, where the main difference was in the “worry” section where parents reported worse QOL. The total scores showed good psychological adjustment of children and adolescents with diabetes, mean score (± SD) was 85.7 (12.45), with slightly worse QOL in the 8-12 year old (71.2±13.1) p<0.05. Growing age, HbA1c, mode of insulin therapy, SES did not influence QOL of children with diabetes. Conclusions: Children and adolescents with type 1 diabetes and their parents in Kuwait showed good psychological adjustment and QOL. Parents appeared to be more worried than their adolescents about the effectiveness of the treatment and the long term complications.

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The effect of a health enhancing physical activity programme on transient ischaemic attack and non-disabling stroke: The methodological design of a randomised controlled pilot trial

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A transient ischaemic attack (TIA) is a brief episode of neurological dysfunction caused by a focal disturbance of brain ischaemia lasting less than 24 hours (Wu et al., 2007). TIA offers a warning sign for subsequent stroke as ~15% of ischaemic strokes are preceded by a TIA (Wu et al., 2007). For patients diagnosed with a TIA, management strategies are predominantly aligned with lifestyle (i.e., advice on smoking cessation, diet) and pharmacological interventions (Manktelow & Potter, 2009).

Coronary Heart Disease (CHD) and Cerebrovascular Disease (CVD) share many predisposing, modifiable risk factors (hypertension, abnormal blood lipids, cigarette smoking, physical inactivity, obesity). Research has shown the benefits of exercise programmes and lifestyle education for individuals with CHD (Joliffe et al., 2010). There is, however, the need to establish and identify the feasibility and efficacy of exercise-based interventions for CVD (Lennon & Blake, 2009; Yang et al. 2010). With mounting evidence highlighting the multifactorial pathogenesis of CVD, an early treatment strategy of the underlying disease process, incorporating physical activity participation, may be an effective approach towards preventing recurrent vascular events. The purpose of the present study is therefore to examine whether a ‘Health Enhancing Physical Activity Programme’ (HEPAP; exercise & education) reduces risk factors aligned with CVD, improves physical fitness, and increases the awareness of educational and health issues associated with TIA.

In this study, 60 patients will be screened for TIA at Wellington Hospital (NZ). All recruited participants will complete a baseline assessment incorporating a risk stratification assessment, exercise ECG stress test, submaximal cycle test and a series of psychosocial lifestyle focused questionnaires (HADS, POMS, SF-36, etc). Participants are then randomized to either an eight week HEPAP (n = 30) or a ‘normal care’ control group (n = 30). The HEPAP will incorporate aerobic and resistance exercises, flexibility and co-ordination / balance-based exercises, with an additional educational component (goal setting, advice on smoking cessation, diet, physical activity and coping with TIA etc), which will be performed in small groups, twice a week (90 min sessions). A further home-based exercise session will also be incorporated into the programme each week. Immediate post-intervention, 3-month and 12 month follow-up assessments will be undertaken (identical to baseline).

This pilot study is ethically approved by the ‘Central Regional Ethics Committee’ and is funded by the Massey University Research Fund. Data collection commenced w/c 14th February 2011, and provisional findings will be presented at the Physiological Society Conference.


Lennon, O., & Blake, C. 2009. Cardiac rehabilitation adapted to transient ischaemic attack and stroke [CRAFTS]: A randomised controlled trial, BMC Neurology, 9, 9.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Improvement in neurocognitive function: disassociating habituation from treatment effects  
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Neurocognitive function assessment (NFA) is commonly used to measure neurologic function. It consists of a battery of psychometric tests designed to challenge memory, mental agility and co-ordination and is traditionally used to assess the effect of a vascular surgical intervention (day before and day after surgery) (Newman et al., 2001; Herman et al., 2003) such as carotid endarterectomy (CEA). However, changes typically recorded post CEA, may be misinterpreted as an improvement when they could actually be the result of a learning effect, possibly even coupled with temporary vascular cognitive impairment. Therefore the aim of the present study was twofold; i) to identify the magnitude of improvement in performance between days 1 and 2 and ii) to determine whether 4 repeated tests are sufficient to achieve habituation.

Twenty healthy volunteers (9♂/11♀) completed a battery of psychometric tests, randomly administered on 4 consecutive days, at a similar time of day. The battery of tests were grouped into 3 subcategories; i) Memory [Ray Auditory Verbal Learning Test (RAVLT), Digit Span Test (RDF + RDB) and the Clock Face Test (CF)]; ii) Mental Agility [Trail Making Tests form A (TMTA) and B (TMTB) and the Digit Symbol Substitution Test (DSST)]; and iii) Visual-motor Coordination [Groove Pegboard Dexterity Test, using dominant (GPD) and non-dominant (GPND) hands]. After confirmation of normality using Shapiro-Wilk W tests, data were analysed using a one-way repeated measures ANOVA and Bonferroni corrected paired samples t-test or the Friedman test and Bonferroni corrected Wilcoxon matched pairs signed ranks t-tests. Significance was set at $P < 0.05$.

The participants significantly improved in 4/9 tests between days 1 and 2 and continued to improve in all mental agility tests, as well as the RAVLT memory test and GPND visual-motor coordination test throughout the 4 tests (Table 1). These findings are the first to suggest that participants continued to improve their performance in mental agility tests, specific memory and visual-motor coordination tests when performed on 4 consecutive days. Therefore, we may in fact be misinterpreting post CEA data. It is suggested that future research needs to consider alternative (i.e non-habituating) biomarkers of neurocognitive dysfunction.

Changes in neurocognitive function performance over the 4 days of testing

<table>
<thead>
<tr>
<th>Test</th>
<th>Day 1-2</th>
<th>Day 1-3</th>
<th>Day 3-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memory</td>
<td>% change</td>
<td>% change</td>
<td>% change</td>
</tr>
<tr>
<td>RAVLT</td>
<td>20.4±3.2</td>
<td>20.4±3.2</td>
<td>20.4±3.2</td>
</tr>
<tr>
<td>RDF</td>
<td>14.2±2.5</td>
<td>14.2±2.5</td>
<td>14.2±2.5</td>
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<tr>
<td>RDB</td>
<td>13.9±3.6</td>
<td>13.9±3.6</td>
<td>13.9±3.6</td>
</tr>
<tr>
<td>CT</td>
<td>1.6±1.4</td>
<td>1.6±1.4</td>
<td>1.6±1.4</td>
</tr>
<tr>
<td>TM</td>
<td>3.2±2.1</td>
<td>3.2±2.1</td>
<td>3.2±2.1</td>
</tr>
<tr>
<td>TMB</td>
<td>11.4±2.6</td>
<td>11.4±2.6</td>
<td>11.4±2.6</td>
</tr>
<tr>
<td>DSST</td>
<td>11.1±6.8</td>
<td>11.1±6.8</td>
<td>11.1±6.8</td>
</tr>
<tr>
<td>PSM</td>
<td>6.4±2.8</td>
<td>6.4±2.8</td>
<td>6.4±2.8</td>
</tr>
<tr>
<td>GPND</td>
<td>3.3±2.0</td>
<td>3.3±2.0</td>
<td>3.3±2.0</td>
</tr>
</tbody>
</table>

Mean ± SD; $P ≤ 0.05$


Comparison of haemodynamics and metaboreflex sensitivity between high intensity interval and constant aerobic exercise in prehypertensive individuals

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Hypertension is one of the most serious clinical risk factors of the cardiometabolic syndrome. Exercise has been shown to reduce arterial blood pressure in normotensive and hypertensive individuals. Although high intensity interval exercise (HIE) has similar or greater benefits to the cardiovascular system compared to constant aerobic exercise (CE), the acute effects of HIE on haemodynamics and metaboreflex sensitivity are less known, particularly in individuals with prehypertension or hypertension. Therefore, we evaluated haemodynamic responses and metaboreflex sensitivity in pre-hypertensive individuals following acute HIE and CE on the treadmill. A total of 13 pre-hypertensive males (systolic arterial pressure; SAP 130±6 mmHg, diastolic arterial pressure; DAP 76±3 mmHg, age 42±4 years, height 170±4 cm, body weight 72.8±7.0 kg, body mass index 25.1±2.1 kg/m2) performed a bout of HIE and CE on a treadmill. CE was performed at 60% of target heart rate (THR) and IE was alternated between 80% THR and 50% THR in the ratio of 1:2 for 30 min, including 3 min warm-up and cool-down for each period. Total exercise calorie expenditures (calculated by respiratory gas analysis) were determined during both exercises (203.8±19.7 kcal for HIE and 205.7±19.3 kcal for CE). Beat to beat SAP, DAP, heart rate and metaboreflex sensitivity were measured noninvasively (Finometer) during 30 min pre and post exercise. Subsequently, dynamic handgrip exercise was performed to measure the metaboreflex sensitivity for 3 min followed by 2 min of postexercise ischemia. Values represent means ± S.D., which were compared by Wilcoxon-signed rank test. R-R interval and stroke volume were decreased by a similar magnitude following both exercises. However, after 30 min recovery period, values of SAP (HIE vs. CE; 114±7 vs. 118±4 mmHg, p<0.05), DBP (72±4 vs. 76±3 mmHg, p<0.05), MAP (89±5 vs. 93±3 mmHg, p<0.05) and total peripheral resistance (TPR: 0.826±0.117 vs. 0.949±0.129 mmHg*s/ml, p<0.05) were significantly lower following HIE than CE. In addition, aortic impedance after HIE was lower (49.1±2.4 vs. 50.1±2.0 milli-mmHg*s/ml, p<0.05), whereas arterial compliance was greater (2.25±0.23 vs. 2.11±0.18 ml/mmHg, p<0.05) compared to those after CE. As compared to resting values, the change (Δ mmHg) in MAP during post exercise ischemia was significantly lower in magnitude following HIE than that following CE (HIE: Δ = 14±6 mmHg, CE: Δ = 20±6 mmHg, p<0.05). Taken together, HIE results in lower arterial stiffness and reduced metaboreflex sensitivity than CE, which suggests that HIE would be more effective in reducing high blood pressure than CE.

PC323

Functional involvement of the calmodulin/inositol 1,4,5-trisphosphate receptor-binding region of TRPC6 in human platelets

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Canonical transient receptor potential channels (TRPCs) have been reported to play an important role in intracellular Ca2+ homeostasis in a number of non-excitable cells, including human platelets. TRPCs contain a conserved calmodulin (CaM)/inositol 1,4,5-trisphosphate (IP3) receptor-binding (CIRB) site in the cytosolic C-terminal region, which binds both IP3 receptor and CaM. The latter has been suggested to be tethered to the TRPCs in a Ca2+ concentration-dependent manner and to be displaced competitively by the activated IP3 receptor (Zhang et al., 2001). In human platelets, TRPC6 are suggested to participate in receptor-activated, diacylglycerol-mediated Ca2+ influx, as well as in store-operated Ca2+ entry (Authi, 2007; Jardin et al., 2009), although little is known about the functional role of the CIRB region of TRPC6 in platelets. Here we have investigated the functional relevance of the CIRB region of TRPC6 in human platelets.

Blood was drawn from healthy volunteers with local ethical committee approval. Cytosolic free Ca2+ concentration ([Ca2+]c) measurement, immunoprecipitation, Western blotting and platelet aggregation were performed as previously described (Redondo et al., 2006; Alexandru et al., 2008). Co-immunoprecipitation studies revealed that treatment of platelets with iminoxymycin in the presence of increasing concentrations of extracellular Ca2+, leading to a gradual increase in [Ca2+]c, results in Ca2+ concentration-dependent dissociation of TRPC6 from the IP3 receptors and association with CaM. Ca2+ concentration-dependent interaction of TRPC6 with IP3 receptors or CaM was impaired by introduction in the cells of a peptide corresponding to TRPC6625-875, containing the CIRB site. Introduction of TRPC6625-875 into cells did not alter the association of TRPC1 or Orai1 with IP3 receptors or CaM, but significantly modified both thapsigargin- and thrombin-evoked Ca2+ entry in a [Ca2+]c-dependent manner, enhancing Ca2+ entry at high [Ca2+]c, while attenuating it at low [Ca2+]c. Furthermore, introduction of the TRPC6625-875 into platelets significantly reduced thrombin-evoked platelet aggregation. These results support a functional role of the CIRB of TRPC6 in human platelets.

Supported by MICINN grant BFU2010-21043-C02-01 and Junta de Extremadura-FEDER GR10010. ND was supported by PRE09020; JLL was supported by a postdoctoral fellowship from the Junta de Extremadura (POS0922).

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PC324

Thapsigargin and the diacylglycerol analogue 1-oleoyl-2-acetyl-sn-glycerol differentially regulate the association between Orai and STIM proteins in human platelets

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Orai1 is a plasma membrane protein that conducts store-operated Ca2+ entry (SOCe), a mechanism regulated by the filling state of the Ca2+ stores, whose depletion can be detected by the sensor protein STIM1. Isomorphs of these proteins, STIM2, Orai2 and Orai3, have been identified, although their role in intracellular Ca2+ homeostasis is less characterized (Roberts-Thomson et al., 2010). In non-excitable cells such as human platelets, physiological agonists stimulate Ca2+ entry via different mechanisms, including SOCE and second messenger-operated Ca2+ entry (SMOCE), activated, in turn, by signalling molecules such as diacylglycerol (DAG) or protein kinase C (Rosado & Sage, 2000; Salido et al., 2009). We have investigated the expression and interaction of Orai and STIM proteins in human platelets.

Blood was drawn from healthy drug-free volunteers with approval of the local ethical committees and in accordance with the Declaration of Helsinki. Platelets were isolated as described (Jardin et al., 2008). Orai and STIM mRNA transcripts were detected by reverse transcription polymerase chain reaction (RT-PCR). Co-immunoprecipitation followed by Western blotting was performed as described previously (Jardin et al., 2008). In resting platelets we detected interaction between Orai1 and Orai2, Orai3, STIM1 and STIM2, as well as between STIM1 and STIM2. In cells loaded with dimethyl-BAPTA by incubation with 10 μM dimethyl BAPTA-AM for 30 min, depletion of the intracellular Ca2+ stores with the inhibitor of the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) thapsigargin (TG) in a Ca2+-free medium significantly enhanced the association between Orai1 and both Orai2 and Orai3 by 49 and 25%, respectively (p<0.05 Students test; n=4). Moreover, TG enhanced the association of STIM2 with either STIM1 or Orai1 to 300% and 219%, respectively, compared to untreated control (p<0.05; n=4). On the other hand, in the presence of 1 mM extracellular calcium, platelet stimulation with the DAG analogue 1-oleoyl-2-acetyl-sn-glycerol, to activate SMOCE, solely enhanced the association of Orai1 with Orai2 and STIM2 by 39% and 85%, respectively (p<0.05; n=4). In conclusion, we report the expression of different Orai and STIM isoforms in human platelets and the differential regulation of their interactions as a consequence of store depletion or DAG signalling activation.


Three nights of sleep restriction impairs forearm skin microvascular reactivity in young healthy males

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Studies indicate a link between sleep duration and increased risk for cardiovascular diseases (CVD) (Cappucio et al., 2011). Endothelial dysfunction, possibly caused by impaired nitric oxide (NO) availability, has been suggested as a potential mechanism linking habitual short sleep duration to CVD (Sauvet et al., 2010). Laser Doppler flowmetry (LDF) to assess the skin blood flow (SkBF) response to thermal provocation provides a simple non-invasive method for evaluating endothelial function in humans (Minson, 2010). Using this approach, we examined the impact of acute sleep restriction (SR) on skin microvascular function. It was hypothesised that skin microvascular capacity would be reduced after SR in comparison to normal sleeping. With local ethical approval and written informed consent, 12 young male subjects (mean ± SEM) completed two randomized tests: one following three nights with normal sleep consisting of eight hours sleep per night (1436 ± 2 min; CON); the other test followed three nights of SR comprising two, one hour bouts of sleep per night (349 ± 3 min). All subjects were healthy, lean, normotensive, and not taking any cardiovascular medication. Energy and fluid provision, and activities of daily living were consistent between tests. On the morning after the third night, following a period of acclimation, cutaneous thermal hyperaemia was assessed on the forearm using an integrating-probe LDF (Periflux 5000). Arterial blood pressures (contralateral arm) and heart rate were monitored at regular intervals throughout the protocol using an oscillometric blood pressure and pulse monitor (Model HEM704C, Omron) Recordings of the laser Doppler signal were made using Perisoft for Windows 9.0 software. Basal SkBF data was recorded for 10 minutes at a standardized skin temperature of 33°C prior to rapid local heating (1°C min⁻¹) to 42°C, which was maintained for 40 minutes. Location of the LDF probe was the same for both tests. Measurements of SkBF (arbitrary perfusion units; APU) were divided by mean arterial pressure (MAP) to give cutaneous vascular conductance (CVC) in APU mmHg⁻¹. Peak plateau (PP) in the SkBF response, which is predominantly dependent on NO, was normalized to baseline (BL) (%CVCBL = [(CVC minus CVCBL)/CVCBL] x 100). Follow-up was lower at 118 ± 115 %CVCBL versus CON; 1579 ± 180 %CVCBL (n = 12, P < 0.05, paired t-test). MAPBL was not significantly different; 86 ± 2 versus 86 ± 1 mmHg. This data suggest that in healthy young males acute SR is linked to impaired NO-mediated vasodilator function. Endothelial dysfunction may, in part, explain high CVD morbidity in persons whose sleep is frequently disturbed and insufficient.


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Artificially Aged Skeletal Myoblasts Display Reduced Regeneration in Bio-engineered Skeletal Muscle

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The degeneration of skeletal muscle (SkM) with age (sarcopenia) is a major consideration for life long health and wellbeing. The extrinsic environment (the skM ‘niche’) has attracted much attention (Conboy et al. 2005) versus the contribution of intrinsic ageing in muscle “stem” cells and their progeny (myoblasts). However, it has become apparent that reduced myotube formation in parental C2 vs. daughter C2C12 myoblasts (Sharples et al. 2010) are associated with key molecular changes during growth and differentiation, which are also altered in artificially aged, serially passaged, myoblasts (unpublished observations). Furthermore, proliferation/differentiation in aged human myoblasts are comparable when cultured in serum from young vs. older humans (George et al. 2010) supporting the notion of intrinsic ageing. To date, studies have been conducted in monolayer cultures, however, to substantiate and extend these findings the current studies utilised an established 3-dimensional (3D) in vitro model (Mudera et al. 2010) to assess artificially aged vs. un-passaged myoblasts capacity to create in vivo-like SkM constructs. Serially passaged C2C12 skeletal myoblasts (63 doublings; passage 20) were compared with un-passaged cells. Cells were seeded at 4×106 cells/ml in type-1 rat tail collagen (3ml) and plated in chamber slides. Floatation bars at either end provided attachment points, enabling the polymerised collagen/myoblast constructs to be suspended in growth medium (20% FBS) for 24hrs prior to transfer to low serum medium (2% HS) to enable differentiation over 3, 7 and 14 days(d). Morphology, immuno-histo logical (desmin) analyses and preliminary transcript changes in matrix metalloproteinases (MMP2, MMP9), myogenic regulatory factors (MyoD, Myogenin), insulin-like growth factor members (IGF-I, IGF-IIEa, MGF, IGF-IR, IGFBP2, IGFBP5) and myostatin were investigated (qRT-PCR). Morphology suggested aged muscle constructs showed reduced ability to attach to the collagen matrix with a 3.3 fold reduction in MMP2 at 7d and a 2 fold decrease in MMP9 at 14d vs. un-aged. This was associated with diminished myotube formation and a 2.6 fold reduction in myogenin in 3d. IGF-I mRNA was reduced 2.1 and 6.3 fold at 3 and 14d respectively together with a 5.1 and 3.8 fold decrease in IGF-IIEa and MGF respectively at 14d. This differences were observed in parallel with 3.5 and 6.6 fold increase in aged cells production of IGFBP8 and 8 and 6.5 fold larger myostatin mRNA levels at 7 and 14d respectively vs. un-aged. This study provides an important 3-D model for studying ageing muscle in-vitro. It consolidates key findings in monolayer cultures of rodent and human muscle cell ageing and further provides important insight into the impact of multiple divisions (artificial ageing), independent of stem cell niche, on the cellular/molecular mechanisms underpinning degeneration. Conboy IM et al. (2005). Nature. 433, 760-764.


Sharples AP et al. (2010). J Cell Physiol. 225, 240-250

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Intracellular calcium and phosphate in intact muscle stimulated to fatigue in the anaesthetised mouse

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Fatigue is the decline in force or power when muscles are used intensively. The mechanisms have been studied in isolated single muscle fibres which are amenable to intracellular measurements. However single fibres lack the restriction of O2 supply and the accumulation of K+ and lactic acid which occur in intact preparations. In an attempt to avoid these deficiencies we studied the intact tibialis anterior muscles of anaesthetised mice (112 mg/kg ketamine + 1.5 mg/kg medetomidine i.p.) with intact blood supply and the distal tendon attached to a force transducer. The muscle was stimulated with electrodes applied directly to the muscle surface and fatigued by repeated (1 per 4 s), brief (0.4 s), maximal (100 Hz stimulation frequency) tetani. Forcedeclined monotonically to 49 ± 5 % (SE, n=13)of the initial value with a half time of 36 ± 5 s and recovered to 86 ± 4 % after 4 min. Intracellular phosphate concentration ([Pi]) was measured by 31P nuclear magnetic resonance on perchloric acid extracts of muscles. [Pi] increased during

Poster Communications

PC327

PC328
fatigue from 7.6 ± 1.7 (SE, n=6) to 16.0 ± 1.6 mmol/kg muscle wet weight and returned to control during recovery. Intracellular calcium was measured with cameleons whose plasmids had been transfected in the muscle 2 weeks before the experiment. Yellow cameleon 2 was used to measure myoplasmic calcium and D1ER to measure sarcoplasmic reticulum (SR) calcium. The myoplasmic calcium during tetani declined steadily during the period of fatigue and showed complete recovery over 4 min. The SR calcium also declined monotonically during fatigue and showed a partial recovery with rest. These results show that the initial phase of force decline is accompanied by a rise in [Pi] and a reduction in the tetanic myoplasmic calcium. We suggest that both changes contribute to the observed fatigue. A likely cause of the decline in tetanic myoplasmic calcium is precipitation of calcium phosphate in the SR.

Supported by the Australian Research Council and the Deutsche Forschungsgemeinschaft (RU 923/3-1). We thank Professor Roger Tsien for plasmids.

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PC330

Kinetic control of oxygen consumption in single Xenopus laevis skeletal muscle fibres is not first-order

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The means to increase muscular oxidative phosphorylation at the onset of contractions is the sine qua non of the ability to sustain exercise. This process is supported in vivo by increasing the delivery rate of substrates for mitochondrial electron transport and ATP synthesis. The mechanisms controlling the adaptation of oxygen consumption (VO2), however, are less well understood. In isolated mitochondria control is well described by a first-order reaction via [ADP], predicting exponential response kinetics [1]. A first-order response, damped by [PCr], also provides a good approximation of VO2 control in recovery from contractions in frog muscle in vitro [2]. We therefore aimed to determine whether a first-order control model could explain VO2 kinetics at the onset and cessation of contractions in single muscle fibres.

For this, single muscle fibres (n=18) were isolated from the iliofibularis of Xenopus laevis, and suspended in oxygenated Ringer’s solution at 20 °C in an 170 μl glass chamber with a fast-response (t=2s) polarographic PO2 electrode [3], [O2] and force production were measured during stimulated isometric contractions and recovery, and then deconvoluted and differentiated to provide VO2 (every 1s). The twitch frequency was chosen to elicit VO2max whilst minimising fatigue. VO2 responses were fitted to a first-order model using non-linear least squares regression to estimate the rate constants (k off and k on). R2 was used to estimate the goodness-of-fit.

The VO2max of type 1 fibres (low oxidative; range, 0.012 to 0.027 nmol.s-1.mm-3) was lower than type 2 (0.046 to 0.130 nmol.s-1.mm-3) and type 3 (high oxidative; 0.118 to 0.133 nmol.s-1.mm-3, P<0.001) [3,4]. While the off-transient VO2 kinetics were well described by an exponential (R2=0.93±0.04) with a mean k off of 1.35 min-1 (range, 0.21 to 2.87 min-1), a first-order response did not provide a good characterisation of the on-transient kinetics (R2=0.83±0.06, P<0.001). Across fibre types the k on was proportional to VO2max (R2=0.80, P<0.001), but was unrelated to k off (R2=0.006, P=0.87). These data show a clear dissociation between the kinetic control of VO2 at the onset and cessation of contractions in single muscle fibres - behaviour inconsistent with first-order control. The significant correlation between k on and VO2max is consistent with the notion that VO2 kinetics are strongly related to [ADP] recovery and mitochondrial volume. However, this phosphate-control of VO2 kinetics may be modulated at exercise onset by the delivery of reducing equivalents or O2, allosteric activation of mitochondrial transporters or enzyme activities [5], and/or a fall in economy. Therefore, more complex models are required to fully understand the processes underlying the activation of mitochondrial oxidative phosphorylation at the start of skeletal muscle activity.


Supported by BBSRC (BB/F019521/1)

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PC331

TNF-α inhibits amino acid uptake and activation of the MAPK pathway in isolated intact mammalian skeletal muscle fibres through a COX-dependent pathway

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Ageing and some chronic illnesses such as AIDS, chronic obstructive pulmonary disease and cancer are characterised by a slow but progressive loss in skeletal muscle mass commonly referred to as sarcopenia and cachexia, respectively. Recent studies suggest that this muscle wasting may arise from the high titres of circulating pro-inflammatory cytokines, especially tumour necrosis factor α (TNF-α; also known as cachexin), associated with both sarcopenia and cachexia (Degens, 2010). However, little is known about the cellular-signal transduction events underlying the acute effects of TNFα in mammalian skeletal muscle fibres. Therefore, the primary aim of this study was to investigate the acute effects of TNF-α and the cellular signal transduction events mediating these effects in isolated intact mouse skeletal muscle fibre bundles.

All the experiments were performed at room temperature (~20°C) using small skeletal muscle fibre bundles isolated from a fast-twitch (edl) and slow-twitch (soleus) muscle of adult male CD1 mice. The mice were killed as recommended by the
Animals (Scientific Procedures) Act 1986, UK and all the experiments conformed to the local animal welfare committee guidelines. The fibre bundles were then treated with either Ringer’s solution only (controls) or the Ringer’s solution plus 200ngml-1 TNF-α (treated). In some experiments, pharmacological interventions were used to investigate the cellular signal transduction events mediating the acute effects of TNF-α in the muscle fibre bundles. At the end of these experiments, the muscle fibre bundles were processed for either liquid scintillation counting or Western blotting.

Our results show that both the fast- and slow-twitch fibre bundles express equal levels of TNF-α receptor 1 protein and that treating them with TNF-α had no effect on the expression of the receptor in both fibre types. However, it led to a decrease in the phosphorylation of ERK1/2, JNK and p38 as well as that of their downstream effectors, especially cJUN. It also led to a significant (<0.05) decrease in the uptake of isoleucine and to an increase in the production of prostaglandin E2α in both fibre types. Furthermore, its effects on the MAPK pathways were partially reversed by pre-treating the muscle fibre bundles with aspirin (a general cyclooxygenase (COX) inhibitor), SC-236 (a selective COX 2 inhibitor) and CAY10526 (a prostaglandin E2α specific inhibitor). From these results we suggest that the catabolic actions of TNF-α in adult mammalian skeletal muscle are mediated through a COX2 dependent inhibition of the MAPK pathway; and that they can be partially reversed by aspirin.


This research was funded by the University of East Anglia

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Dihydrotestosterone (DHT) stimulates amino acid uptake in mouse fast-twitch fibre bundles by increasing the expression and activity of the L-type amino acid transporter (LAT) 2

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In a recent communication to the society we showed that dihydrotestosterone (DHT) stimulates amino acid uptake in isolated intact mouse fast-twitch skeletal muscle bundles without significantly affecting uptake in slow-twitch fibre bundles (Mutungi & Hamdi 2010). This effect, like that which DHT had on force (Hamdi and Mutungi, 2010), was mediated through the epidermal growth factor receptor (EGFR) and involved activation of the mitogen-activated protein kinase (MAPK) pathway. However, little is known about the amino acid transporter(s) stimulated by DHT. Therefore, the primary aim of this study was to investigate the amino acid transporter(s) mediating DHT-induced increase in amino acid uptake in mouse skeletal muscle fibre bundles.

All the experiments were performed at room temperature using small fibre bundles isolated from a fast-twitch (edl) and slow-twitch (soleus) muscle of adult female CD1 mice. The mice were killed as recommended by the Animals (Scientific Procedures) Act 1986, UK and all the experiments conformed to the local animal welfare committee guidelines. The fibre bundles were treated with Ringer’s solution containing either 107.9μM ethanol (controls) or 630μgml-1 DHT (treated) plus 2mM carbon-14 labelled isoleucine (L-(U-14 C) Ile; 3.46μCi ml-1) for 1hr. In some experiments, pharmacological interventions were used to investigate the transporter(s) mediating the effects of DHT on L-(U-14 C) Ile uptake. At the end of the experiments, the fibre bundles were processed for either liquid scintillation counting or Western blotting.

Treatment of the muscle fibres with DHT increased the uptake of both isoleucine (Ile) and α-methylaminoisobutyric acid (MeAIB) in the fast-twitch fibre bundles only. Also, it increased the expression of the L-type amino acid transporter LAT2 and led to an increase the phosphorylation of the epidermal growth factor receptor (EGFR) in the same fibre bundles and that of RSK1/2 in both fibre types. However, it had no effect on the phosphorylation of the mitogen- and stress-activated protein kinases (MSK) 1/2. Pre-treating the fibre bundles with MeAIB (a system A amino acid transporter inhibitor) and 2-amino-3-methylaminoisobutyric acid (BCH; a system L amino acid transporter inhibitor) significantly (p<0.05) reduced the basal uptake of Ile in both fibre types and completely abolished the DHT-induced increase in Ile uptake. From these findings we suggest that DHT stimulates amino acid uptake in isolated mammalian skeletal muscle fibres by modulating the activity of LAT2 and the sodium-coupled neutral amino acid transporter (SNAT) 2. These effects are mediated through the EGFR and involve an increase in LAT2 expression.


This research was funded by the University of East Anglia. MMH is a PhD sponsored by the Malaysian Government.

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A Putative Model of Endurance Exercise Using Bio-Engineered Skeletal Muscle

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The metabolic flux with in vivo exercise, including increases in Lactate production and Glucose uptake, have been used in exercise physiology to characterise bouts of exercise with respect to the intensity and energy systems involved. The use of in vitro skeletal muscle models for physiological testing can allow for greater amounts of experimentation and control compared to in vivo investigations. Models of skeletal muscle in vitro which respond physiologically will allow for insight into the cellular and molecular mechanisms involved in the adaptation of skeletal muscle following exercise. This investigation aimed to characterise a 3D in vitro skeletal muscle model (Mudera et al., 2010) with respect to endurance exercise. 4x106/ml C2C12 mouse muscle cells were seeded in 3ml of type-1 rat tail collagen and plated into chamber slides (n=3). Each chamber held an “A-frame” at either end to provide attachment points. Once set the collagen construct was cut away from the sides of the chamber and suspended in growth medium (20% FBS). The “A-frames” provided tension to allow for the development of myotubes. The constructs were left in culture for 14 days for optimal myotube development. The
construct was then tethered to the tensioning culture force monitor (t-CFM) for mechanical stimulation. The protocol was as follows: 7.5% strain, cyclic continuous stretch at 0.4Hz for 60 minutes. Conditioned media was sampled every 10 minutes throughout for analysis. Control samples were tethered to the t-CFM without stretch. An Analox Analyser was used to analyse both [Lactate] and [Glucose] within the conditioned media. Following stimulation the construct was immediately sampled. qPCR was performed using primers for β-Globin (Nuclear encoding) and COXII (Mitochondria encoding). mtDNA copy number was represented per nuclear diploid genome. 60 minutes of cyclic stretch increased [Lactate] at every time point compared to 0 minutes from 1.83±0.23mmol.L to 4.52±0.21mmol.L (p<0.05). There were no significant differences between each time point after 0 minutes (p>0.05) indicative of a ‘steady state’ in Lactate production. The index of uptake ([Glucose] at 0 minutes - [Glucose] at experimental time point) of Glucose was significant after 30 minutes of the stretch protocol from 0mmol.L to 1.5±0.12mmol.L (p<0.05). There were no changes in Lactate or Glucose for controls. mtDNA copy number per nuclear diploid genome increased compared to control immediately post stretch (p<0.05). This model of mechanical stretch (‘exercise’) has shown in vivo-like responses with respect to both Lactate and Glucose metabolism. This is the first investigation to show an increase in mtDNA copy number following an acute bout of mechanical stimulation in vitro. This model will allow for future investigations to understand the cellular and molecular adaptation to acute exercise.


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**PC334**

Effect of hindlimb suspension on the expressions of neurotrophins in rat soleus muscle

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The aim of this study was to examine the effect of hindlimb suspension (HS) on the expressions of neurotrophin (NGF, BDNF, NT-3) proteins and their receptors (TrkA, TrkB, TrkC, p75) in rat hindlimb muscles. Skeletal muscle shows the plasticity against changes in the mechanical environment. Decreased physical activity leads to muscle-fiber atrophy and muscle-fiber type shift from oxidative to glycolytic. HS has been reported to induce changes in muscular properties mainly in the slow muscles such as soleus muscle, in similar manner to space flight or simulated micro gravity (Edgerton & Roy, 1998; Ohira, 2000). Neural system as well as muscle fibers has been shown to be affected by HS. The afferent input from proprioceptors in steady state transitionally decreases after HS (Kawano et al. 2004). In another study, the afferent discharge of reflex was reported to increase during HS (De-Doncker et al. 2003). Neurotrophins are family of members of proteins which induce the survival, development and function of neurons. In the present study, we hypothesized that HS would affect the expressions of neurotrophines and their receptors which are assumed to affect the neural potency in soleus muscle.

5-week-old male Wistar rats (n=32) were divided into HS (1 week; n=8, 2weeks; n=8) and control (1 week ;n=8, 2weeks; n=8) groups. During HS rats were suspended with their tails and free to access to food and water on their forearms. Rats were monitored via behavioural observations in addition to monitoring for general physical health. After 1 and 2 wk of HS, rats were anesthetized with isoflurane and killed by blood removal to excise soleus from both legs. After homogenizing soleus in RIPA buffer, westernblotting was performed to examine the expressions of NGF, BDNF, NT-3 proteins and TrkA, TrkB, TrkC, p75 receptors. Immunohistochemistry was examined to determine the loci of those proteins and receptors during HS.

During the exposure to HS, NGF, BDNF, NT-3 and TrkA, TrkB, TrkC, expression decreased in the soleus muscle at day 7 and 14, except p75. P75 content interestingly increased during HS. The signals of immunohistochemistry of NGF and BDNF were located at motorneurons in soleus muscle. The immunohistochemistry of NT-3 and TrkC revealed their expressions around nerve-related tissues including muscle spindles and connective-tissues sheath. Localizations of neurotrophins were not altered after HS.

The results indicate that the protein contents of neurotrophins and Trk receptors in the soleus muscle decrease in response to unloading. Mechanical stress affects the NT-3 and TrkC production, which may induce the change of function of muscle spindle. However, p75 protein abundance increased through HS, which might cause a different effect on skeletal muscle atrophy by mediating apoptosis. It is concluded that neuromuscular activities may be important for maintaining metabolism of neurotrophins which regulate the functions of efferent and afferent neuromuscular systems.


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**PC335**

Relationship between serum pH and seizure onset following birth asphyxia in the newborn

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Hypoxic-ischemic encephalopathy (HIE) is due to perinatal asphyxia causing morbidity and mortality. It affects 2-3 newborns per 1000 live births. (1) Asphyxia is characterized by hypoxia around birth leading to an increase in [H+] in the blood, and acidosis. (2) HIE can be graded depending on the severity and the physiological effects. Grade 1 is mild, Grade 2 moderate and Grade 3 severe. (3) Seizure activity is detected using continuous video EEG monitoring. (4) Studies in rat models suggest that a rapid restoration of pH from acidic levels following asphyxia to alkalotic levels leads to enhancement of neu-
ronal excitability resulting in an increase in seizure activity.(5) The aims were;

i. To establish the range of serum pH values at seizure onset in the human newborn.

ii. To examine the time to normalization of serum pH between newborns with and without seizures.

A retrospective chart review of newborns with HIE was undertaken. The pH results, seizures, medications, and EEG results were recorded for each newborn. The range of normal pH was considered to be 7.35-7.45 where acidosis was <7.35 and alkalosis was >7.45. Statistical analysis was performed using PASW Statistics 18.0, using the Mann-Whitney U test. A p-value <0.05 was considered to be statistically significant. Twenty four full term newborns; 12 with seizures and 12 with no seizures. Seizure newborns had an initial pH value that was in the range of 6.90-7.396. EEG seizure activity was recorded within the 7.35-7.45 range for ten newborns while one newborn had a seizure at 7.17 and the other had a seizure at 7.465. Eight suffered a Grade 2 HIE and four suffered a Grade 3 HIE. All newborns survived. The non-seizure group had an initial pH range of 6.32 to 7.326. From this group, there were eight newborns with Grade 2 HIE and four with Grade 3 HIE. Two newborns died. The initial median pH value of the non-seizure group was 7.03 (IQR:6.74-7.19) while the seizure group had a median pH value of 7.15 (IQR:7.03-7.21). The median initial pH values between the groups was not statistically significant (p=0.141). The non-seizure group had a median time to pH normalisation of 4.47 hours (IQR:3.09-8.58) while the seizure group had a median time of 10.23 hours (IQR:4.75-21.75) but the time to normalisation was statistically significant (p=0.039). The time to the first normal pH had a median value of 10.27 hours (IQR:4.91-21.69) and the time to first EEG seizure onset had a median value of 17.97 hours (IQR:12.2-25.5). The time to pH normalisation and seizure onset in the seizure group was not statistically significant (p=0.158). Seizure newborns remained acidic for longer compared to non-seizure and subsequent seizures were seen with the onset of pH normalisation. The onset of seizures occurs at the same time as pH normalisation. This implies that the normalisation of pH to normal values leads to seizure activity.


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Effects of ghrelin administration on regulation of food intake in arthritic rats

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Adjuvant-induced arthritis is an animal model of chronic inflammation and rheumatoid arthritis. Arthritis induces cachexia that leads to loss of body weight, muscle atrophy and anorexia. Ghrelin is a circulating orexigenic hormone secreted from the gastrointestinal tract that is involved in the control of food intake and energy balance. Peripheral administration of exogenous ghrelin has been shown to increase food intake and body weight in rats. Aim: To analyse the effect of ghrelin administration on food intake regulation in arthritic rats. Methods: Arthritis was induced in adult male Wistar rats by an intra-dermal injection in the sole of the right paw of 4 mg of complete Freund’s adjuvant. On day 6 after adjuvant injection, control and arthritic rats were treated with ghrelin (50 nmol/kg sc) twice a day, until day 15 when all rats were humanly killed. Arthritis induces anorexia, for that reason we included a paired group. Gene expression of leptin receptor and orexigenic peptides: neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the hypothalamus were quantified using RT-PCR. Serum leptin was measured by radioimmunoassay. Results: Arthritis decreased body weight and food intake (P<0.01), but increased AgRP gene expression in the hypothalamus (P<0.01) and decreased serum leptin levels (P<0.01). In control rats, ghrelin administration increased food intake, the two first days of treatment, and body weight (P<0.05). Ghrelin administration increased serum leptin levels in control rats (P<0.05) but not in arthritic rats. In contrast, in arthritic rats ghrelin increased the expression of NPY and AgRP mRNA in the hypothalamus (P<0.01). Conclusion: Although in arthritic rats ghrelin treatment stimulated the expression of orexigenic peptides in the hypothalamus, arthritis seems to induce resistance to the orexigenic action of ghrelin.

This work was supported by grants FIS PS09/00753, BFI06.31 to E Castillero and BES-2007-16001 to M López-Mendúeña.

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Fenofibrate administration ameliorates the inhibitory effect of adjuvant-arthritis on leptin and adiponectin

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Fenofibrate, a PPAR-alpha agonist prescribed to treat human dyslipidemia, has anti-inflammatory effects and improves arthritis-induced skeletal muscle wasting. Adiponectin is exclusively secreted by adipocytes and exhibits metabolic and anti-inflammatory properties. Aim: To elucidate the effect of fenofibrate administration on adiponectin and leptin secretion in arthritic rats. Methods: Arthritis was induced in male Wistar rats by an intradermal injection of 4 mg Freund’s adjuvant. Three days after the injection control and arthritis rats were
The role of clock genes and clock controlled metabolic genes in the developmental priming of fatty liver disease

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Sub-optimal nutrition during early development is associated with adult onset of the Metabolic Syndrome (MetS), the hepatic manifestation of which is Non-Alcoholic Fatty Liver Disease (NAFLD). We have previously shown that high fat (HF) exposure during early development primes the development of non-alcoholic steatohepatitis (NASH – severe NAFLD) in adulthood through elevated lipogenesis and mitochondrial dysfunction (1). Since both fatty acid and mitochondrial metabolism are regulated by the endogenous molecular “clock” network, we hypothesised that “clock” perturbations could modulate metabolic output and contribute to developmental priming of NASH. Therefore, we investigated whether developmental HF exposure could induce day and night changes in NAD+/NADH levels, and may contribute to hepatic clock and clock-controlled gene expression pattern. Specifically, combined HF exposure during development and adulthood appears to increase expression of SIRT1, which may contribute to the observed deregulation of PER2 and Rev-Erbβ. In addition, we suggest that reduced SIRT1 in the HF/HF offspring vs. C/C, which may contribute to the observed deregulation of PER2 and Rev-Erbβ.

Supported by BBSRC and BHF

Poster Communications

Effect of duration-of-stay at high altitude on plasma hormones and psychological variables

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Acclimatization to hypobaric hypoxia of high-altitude (HA) is a complex process. Endocrine changes precede and bring about the necessary physiological changes. Lowlanders exposed to the ‘new’ HA environment also experience psychological changes - a stress that is known to affect plasma hormone concentrations. The aim of the study is to examine: plasma hormone and psychological changes in lowlanders during short- (1-month) and prolonged-stay (6-month) at HA in comparison to sea-level, and with HA-natives at HA. Correlation between psychological variables and plasma hormone levels at HA was also examined. Subjects were male volunteers between 20-50 years of age. Lowlanders (n=25) were studied at sea-level, after 1-month stay at ~4500m, and after 6-month stay at ~3600m. HA-natives/Ladakhis (HAN, n=21) were studied at HA only. Psychological tests include: Memory(Digit-symbol); Depression(Beck-Depression-Inventory); Hopelessness(Beck-Hopelessness-scale); Loneliness(UCLA-Loneliness-scale). Plasma hormone levels were measured by enzyme immunnoassay/enzyme-linked immunosorbent assay. Short-stay at HA causes changes in certain plasma hormone levels (cortisol, prolactin, T3, T4) and is associated with depression, loneliness and fear-of-death. Further stay at HA results in an increase in plasma T4. Memory, hopelessness and plasma TSH remain unaffected by HA.
Galanin and different levels of energy requirements on gonadotropins levels in female goats

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Researchers intended to find out the underlying mechanism of the link between energy status and reproductive function. Galanin is an orexigenic agent and has been demonstrated as putative regulator of gonadotropins secretion. It is well known that orexigenic peptide probably play the agent role in the regulation of reproduction. The goal of this study was to determine the role of galanin in regulation of gonadotropins on diet that was different in energy status. Adult female saanen goats were randomly divided into 2 groups. Animals in the first and second groups were fed 100 and 150% energy content in diet respectively for a month. First day of the experiment, all animals were received 1 ug galanin/kg body weight into their jugular vein. On the second day, they were treated with 2 ug galanin/kg body weight. Blood samples were withdrawn from the jugular vein at 30-minute intervals, 3.5h before and 3.5h after injection by jugular vein puncture. In order to determine the concentration of LH and FSH, plasma was isolated. RIA was applied to measure hormone concentration of gonadotropins. Two-tailed paired t test was used to compare the regulation of reproduction. The goal of this study was to determine the concentration of LH and FSH, plasma was isolated. RIA was applied to measure hormone concentration of gonadotropins secretion in saanan goats. Infact galanin may not be considered as a main hypothalamic regulator of gonadotropins secretion in saanan goats. Infact galanin may negatively affects mean plasma concentration of LH and FSH in goats particularly with positive energy balance. Baratta M & Saleri R & Maccaridi C & Coy D H & Negro-Vilar A & Tamamini C & Giustina A (1997). Neuroendocrinol 66,271-277.

Where applicable, the authors confirm that the experiments described here conformed with The Physiological Society ethical requirements.
Impaired kidney function in rats six months after unilateral nephrectomy

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The development of kidney transplantation has made great strides in the therapy of end stage renal disease (ESRD). It has provided better quality of life (1) and improved survival of patients with ESRD compared with the outcomes of patients on dialysis treatment (2). The shortage of cadaver kidney donors and simultaneously increased demand for kidney transplantation has recently led to a rise in living-donor kidney donation (3,4). However, data on the consequences and its impact on the quality of life and health of living-donors are scarce. The aim of the present study is to examine the long-term changes and function of the remnant kidney after unilateral nephrectomy in an animal model. Here we present our preliminary data on serum creatinine, sodium and potassium levels, and haemoglobin content in red blood cells after the first 6 months of nephrectomy.

36 female Sprague-Dawley rats were randomized into three groups: unilaterally nephrectomized (A), sham-operated (B) and naïve rats (C). In nephrectomized rats (anesthetized by an i.p. injection of 75 mg/kg of ketamin (Ketanest S 25 mg/ml, Pfizer) and 0.5 mg/kg of midazolam (Midazolam Torrex S 5 mg/ml, Torrex Chiesi Pharma)) a small lumbar incision was made, and the left kidney was removed. In sham-operated animals, the left kidney was exposed and gently manipulated but left intact. The blood was taken by incision of the tail vein at the inclusion in the protocol and after six months. The data were analysed by Wilcoxon’s or Kruskall-Walis test (SigmaPlot 11.0) and considered significant if p<0.05.

Initial serum creatinine concentration did not vary among any of the groups (p=0.935). There is a significant increase in serum creatinine concentrations after 6 months in unilaterally nephrectomized rats (39.7±0.8 μmol/l, n=9) compared with sham-operated (30.1±1.1 μmol/l, n=9) and naïve rats (26.3±3 μmol/l, n=5, p=0.001) (Fig. 1). The serum sodium levels remained the same (p=0.116). Blood haemoglobin content did not differ among the three groups of rats (p=0.115). However, 6 months after the introduction to the study serum potassium level was significantly decreased in sham-operated rats (p=0.006).

Although it has been very well established that a kidney possesses a huge capacity to compensate for severe loss of renal mass (5), our results implicate that these reserves can decline with aging. However, reduced function of the remaining kidney found 6 months after nephrectomy does not affect sodium, potassium or haemoglobin levels to any significant extent. In everyday clinical practice we do not see severe renal dysfunction in patients with a single kidney, but in the future prolonged life span and use of more specific biomarkers could support our findings.

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Tissue-specific responses of human uteroplacental smooth muscles to Ca²⁺/calmodulin-dependent kinase II inhibition

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Successful development of the human fetus during pregnancy depends on tight regulation of the uterine and placental vasculatures in order to allow appropriate exchange of oxygen and nutrients from the mother to the placenta. We have previously identified differences in modes of regulation of myometrial versus placental blood vessel function (1, 2) that may be indicative of generic differences in the behaviour of smooth muscle-rich tissues of the human uteroplacental environment. Decoding of the Ca²⁺ signals that underlie smooth muscle contractility may involve not only the archetypal pathway of Ca²⁺/calmodulin-dependent myosin light chain kinase activation but also ancillary Ca²⁺-sensitive kinases, such as the multifunctional Ca²⁺/calmodulin-dependent kinase II (CaMKII). Therefore, the aim of this study was to compare the relative effect of pharmacological inhibition of CaMKII on contractil-
Reactive oxygen species potentiate vasodilator effect of nitric oxide donor with gold nanoparticles in aortas from renal hypertensive rats

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Nitric Oxide (NO) plays important role in the control of the vascular tone, but it can be degraded by reactive oxygen species that are higher in renal hypertensive rat (2K1C) aorta and that are higher in renal hypertensive rat (2K1C) aorta and can be modulated by reactive oxygen species that are higher in renal hypertensive rat (2K1C) aorta and that are higher in renal hypertensive rat (2K1C) aorta. Our results demonstrate that although PE induced similar contraction in 2K and 2K1C aorta, the vasodilatation induced by AuNPs-[Ru-4PySH]n was more potent in 2K1C than in 2K. It seems that ROS potentiate the vasodilatation induced by AuNPs-[Ru-4PySH]n. Supported by FAPESP, CAPES and CNPq

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PC344

Role of Kv1.3 channel in vascular smooth muscle cells proliferation in a porcine model of coronary restenosis

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Vascular smooth muscle cells (VSMCs) regulate vessel diameter and determine tissue perfusion, however mature VSMCs are not definitively differentiated and have the ability to switch from a contractile phenotype to a proliferative phenotype. The phenotypic switch implies a profound change in gene expression profile which includes ion channels expression. Due to the large molecular diversity of ion channels, changes in their expression during phenotypic modulation are poorly defined. In a previous work (1) we performed a high-throughput real time PCR screening of ion channels genes as to obtain a global picture of ion channel expression in proliferation. Changes in mRNA and functional expression showed that Kv1.3 channel had a significant role in the phenotypic switch. In this work we try to reproduce these results in a more clinically relevant VSMC
proliferation model: porcine coronary proliferation. This model closely resembles the human model and could be suitable for assaying in vivo strategies to inhibit local VSMCs responses after surgical procedures. Animal protocols were all approved by our institutional Care and Use Committee, and are in accordance with the European Community guiding principles. Animals were sedated with intramuscular injection (2 mg/Kg) of azaperon and anesthetized with 30 mg/Kg of intravenous sodium thiopental. Coronary intimal lesion was induced by using a guiding catheter from the right femoral artery to the circumflex and the anterior descending coronary arteries, right coronary arteries were used as control. Four weeks after the surgery the animals underwent euthanasia and arteries samples were collected. mRNA expression levels studied by real time PCR showed that accordingly with previous data, Kv1.3 expression increased in injured arteries, when compare to control arteries. By electrophysiological techniques we explored the functional contribution of Kv1.3 in freshly dispersed VSMCs obtained from injured and control arteries. Our data showed that Kv1.3 currents became predominant K+ currents in proliferative VSMCs. Moreover proliferation assays showed that Kv1.3 current is an essential component to the proliferative phenotype as VSMCs proliferation was inhibited in presence of Kv1.3 blockers (10 mM Margatoxin and 10 mM PAP-1), but not in the presence of another Kv channels. These data suggest that Kv1.3 could be a good therapeutic target to treat restenosis in a good translational animal model, as it is porcine coronary arteries, for testing medical devices such as coated stents with Kv1.3 blockers.

Cidad P. et al. (2010). Arterioscler Thromb Vasc Biol., 30:1203-1211. This work was supported by grants from the Spanish Ministerio de Sanidad, ISCIII (R006/009, Red Heracles), Ministerio de Ciencia e Innovacion (BFU2007-61524 and BFU2010-15898) and Generalitat de Catalunya (CIDEM-VALTEC09-1-0042).

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PC346

Kv7.4 channels contribute to β-adrenoceptor regulation of rat renal artery

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KCNQ-encoded voltage-dependent potassium channels (Kv7) have been identified in vascular smooth muscle cells and shown to regulate vascular tone (Greenwood and Ohya, 2009). Kv7.4 channels have been identified as one of the main functional contributors in vascular tissue. Although modulation of these channels with selective activators and blockers causes profound changes in vascular tone, it is not known to what extent Kv7.4 channels contribute to endogenous dilator mechanisms. The present study utilises Kv7 modulators and a selective Kv7.4 knockdown protocol to characterise the impact of these channels in β-adrenoceptor-mediated relaxation of rat renal artery. Renal arteries were isolated from male Wistar rats (200-225 g). Isometric tension recordings were performed on a myograph. In some vessels, Kv7.4 channel knockdown was achieved by transfecting with specific KCNQ4 siRNA using a reverse permeabilisation protocol. Control vessels were transfected with non-specific scrambled siRNA. Subsequent experiments were conducted following 72 hrs incubation. Kv7 channel blocker (linopidine) and activator (S-1) were used to assess Kv7 channel functionality. Furthermore, responses to isoprenaline (β-adrenoceptor agonist) and forskolin (adenylyl cyclase activator) were determined in arteries preconstricted with 3 μM Methoxamine, in the presence and absence of various Kv+ channel blockers. KCNQ gene expression was determined using endpoint and quantitative PCR. Kv7.4 channel expression was assessed by Western blotting. Values are presented as mean ± s.e.m., compared by two-tailed Student’s t-test. Amplitcins of KCNQ1, KCNQ3, KCNQ4 and KCNQ5 mRNA as well as Kv7.4 protein were detected in rat renal arteries. Application of the Kv7 activator S-1 caused concentration-dependent relaxation in renal arteries, which was inhibited in the presence of linopidine. In preconstricted arteries, isoprenaline-induced relaxation was inhibited in the presence of 10 μM linopidine (Emax, 39.3±15.5 vs. 103.6±4.2 vehicle control; n=5-6; p<0.01). The Kv channel blocker 4-aminoptyridine (1 mM) or KATP blocker glibenclamide (10 μM) did not inhibit isoprenaline responses. Relaxation caused by forskolin was also attenuated in the presence of linopidine (pEC50, 6.3±0.1 vs. 7.2±0.1 vehicle control; n=4; p<0.01). In arteries transfected with KCNQ4 siRNA, S-1 relaxation was attenuated (1 μM S-1: 17.2±5.8 vs. 43.5±9.2 in control vessels; n=7; p<0.05). Moreover, in these arteries, relaxation in response to isoprenaline was significantly attenuated compared to control vessels (pEC50, 7.1±0.1 vs. 8.2±0.1 scrambled siRNA control; n=7; p<0.0001). These results show that Kv7.4 channels appear to be functional contributors to β-adrenoceptor-mediated relaxation.


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PC347

The effects of gestation and agonists on the ability of magnesium to reduce uterine contractility

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Magnesium is able to inhibit smooth muscle contractility; an effect attributed to inhibition of L-type Ca channel entry, and as such is used as a uterine tocolytic. Although it is cheap and readily available, its efficacy has been seriously questioned. We have therefore examined two likely variables on its efficacy; stage of gestation and hormonal background, as little is known about how these may influence the myometrial response to increasing [Mg]. Myometrium was dissected from non-pregnant, day 14 and day 22 pregnant rats and from biopsies from women having term, elective Caesarean sections (with consent and ethical approval). Tissues were superfused with physiological saline, Ca 2 mM, pH 7.4, temperature 37 oC and tension measured. Magnesium sulphate was incrementally increased in this solution from 1.2 up to 15 mM and effects on contraction amplitude, frequency and area under the curve (auc) determined. In some experiments oxytocin (1nM, human, 0.5nM rat) or high-K (40mM) were used. Elevation of [Mg] produced a dose-dependent inhibition of uterine spontaneous activity. In non-pregnant and day 14 preg-
nant rats, force was abolished when Mg was raised to 2.0 mM Mg, n=5. Elevation of external Ca to 4-5 mM restored contractions to control levels. In contrast, in term pregnant myometrium of rats and women, at 2.0 mM Mg, force amplitude (control =100%) was reduced but not abolished: 60±10%, n=14, and 43±17% n=7, respectively. Similar reductions were found in the other parameters of contraction. Contractions were abolished at 2.6 mM in all preparations. Application of oxytocin significantly increased the amount of Mg required to affect contractions in term myometrium, such that 2 mM was without effect and to abolish contractions 10-14 mM were required. Similar preliminary results were obtained with high K stimulation.

We conclude that (i) the myometrium irrespective of species, gestation and mechanism of stimulation is sensitive to Mg in a dose-dependent manner but (ii) changes associated with the transition to labour reduce its effectiveness. Thus, variations between women in progression to labour could explain why it is not a reliable tocolytic in vivo. Magnesium’s action appears to be via L-type Ca channel inhibition and measurements of intracellular Ca are on-going to test this further.

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PC348

Caveolin-1 knockout reduces the expression of cavin-1, but not flotillins, in murine smooth muscle

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Caveolae are associated with the orchestration of multiple signalling pathways in most mammalian cells including smooth muscle. However, there is considerable debate as to which proteins are critical components of caveola formation, integrity and function. Caveolin-1 has long been known to serve these functions. Recently, expression of cavin-1 (also known as PTRF) in heterologous cells has been found to induce formation of caveolae in a caveolin-1-dependent manner [1]. Furthermore, cavin-1 knockout mice show much reduced levels of caveolin-1, cavin-1 and no appearance of caveolae in a number of tissues [2].

In mice, cavin-1 or flotillin-2 proteins were altered in smooth muscle tissue of caveolin-1 knockout mice. As expected, devoid of caveolin-1 (and caveolae as examined by electron microscopy) but they also showed significantly reduced levels of cavin-1 (cavin-1 expression was 32.3 ± 10.1 a.u. versus 0.88 ± 0.39 a.u. in WT and KO respectively, mean ± sem, n=6, P<0.05, students unpaired t-test on log transformed data). In contrast, neither flotillin-1 (WT 24.3 ± 3.10 a.u. versus KO 19.8 ± 3.22 a.u.) nor flotillin-2 (WT 47.3 ± 1.63 a.u. versus KO 39.8 ± 3.55 a.u.) protein expression was significantly altered by caveolin-1 KO. We conclude that caveolin-1 ablation concomitantly reduces the expression of cavin-1, but not flotillin-1 or -2, in murine smooth muscle. This supports the possibility that the reciprocal regulation of caveolin-1 and cavin-1 proteins may be an important feature of caveola function in smooth muscle.


Supported by BHF.

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PC349

The Novel Potassium Channel Kir7.1 is a Critical Component of Uterine Quiescence in Mice and Human

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A genome-wide screen of myometrial smooth muscle (MSM) mRNA expression revealed a novel channel (Kir7.1, KCNJ13) that displays biophysical properties suited to mediating uterine quiescence. Computer simulations predicted that Kir 7.1 could be a critical regulator of myometrial cell excitability. To address this, myometrial biopsies were taken in women at the time of CSection (PTL 28-32 Wks, T 37-40wks NIL n=8, TLAB, n=8) and in mice GD13-18 (n=5/GD). qPCR was utilized to determine gestation dependent expression. Protein localisation was determined by immunohistochemistry. In vitro force recordings before and after administration of the inhibitor, VU590 determined the functional contribution of Kir7.1. Membrane potential (Vm) was determined by administration of VU590 under current clamp conditions using microelectrodes. In vivo contractility was tested by direct injection of VU590 to uterine horns of pregnant mice (n=3).

In mice, KCNJ13 mRNA increased 30-fold (G13 vs GD15, P<0.001) to a peak GD15 followed by a 20-fold decline to term (GD15 vs GD18, P<0.001). In human TNIL and TLAB were >4-fold less when compared to PTNIL (Student’s t-test: P=0.049 and 0.042 respectively). In mice and human, IHC demonstrated specific staining for MSM.

VU590 stimulated a dose dependent increase in tone as a percentage of control in both GD 15/18 mouse myometrium, this increase is significantly greater in GD15 than GD18, as would be expected due to the 20-fold increase in mRNA expression. OT elicited a significant increase in tone in GD 15 + 18 myometrium (438.1 ± 95.3% and 806.7 ± 153.8% respectively, however when OT was co administered with VU590 10 μM a substantially greater increase in tone was recorded (17294.4 ± 1457.0% and 8825.2 ± 4309.8%). Application of 100 μM VU590 to human myometrium stimulates an immediate contraction, typically > 20 min duration. Application of 1 μM VU590 to mice and human stimulated contraction followed by an enhancement of contractile force without the tonic com-
Effect of high external potassium on spontaneous calcium oscillations in isolated ICC from the rabbit urethra

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The rabbit urethra contains interstitial cells of Cajal (ICC) which are non-contractile and spontaneously active showing regular propagated intracellular calcium waves. The frequency of these is modulated by calcium influx across the cell membrane and increased when external potassium concentration is increased. The purpose of the present study is to elucidate the mechanism of this response. Urethra was removed from rabbits killed by lethal injection of pentobarbitone. Strips were dispersed in Ca²⁺-free solution containing collagenase. Smooth muscle cells (approx 95%) and ICC (5%) were released. These were loaded with fluo-4/AM and measurements made of intracellular Ca²⁺ using fast confocal microscopy. When [K⁺]o was increased from 5.9 to 60 mM (by replacing NaCl with KCl) frequency of calcium waves in ICC increased from 5.6 ± 0.85 min⁻¹ to 11.65 ± 1.33, mean ± SEM, n=33. It is known, however, that decreasing [Na⁺]o can of itself increase frequency of spontaneous waves so, to control for this, Na was replaced by KCl and depolarisation maintained until the drug was thoroughly washed out. Injection of 100 μM VU590 directly into the uteri of pregnant mice (GD15-18) induces an immediate and tonic contraction (n=3).

In conclusion Kir7.1 is critical for uterine quiescence. Computer simulations predict a two-fold importance. Firstly, in maintaining Vm below threshold and secondly in mediating AP spiking frequency and hence force during a contraction.

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expressing the NaV1.5 α subunit. RT-PCR was used to probe for transcripts of Na+ channels in both whole bronchi and groups of 10–20 isolated ASM cells. Transcripts for NaV1.2 and NaV1.5 were found in ASM (5 replications) and those for NaV1.2, NaV1.3, NaV1.4, NaV1.5, NaV1.8 & NaV1.9 in the whole bronchus. These data show that rabbit ASM express a voltage-gated Na+ current with fast kinetics, which is relatively insensitive to TTX. The evidence suggests that most of this current is conducted by channels expressing the NaV1.5 isoform, although other subtypes may also contribute. More work will be necessary to determine the role of this current in health and disease.


Caveolar disruption with methyl-β-cyclodextrin (MCD) causes endothelium-dependent contraction of rat femoral arteries

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Caveolae are flask shaped invaginations in the cell membrane which act as signalling hubs in endothelial and smooth muscle cells. Disruption of caveolae by the membrane cholesterol depleting agent MCD has various functional effects on arteries, including impairment of endothelium-dependent relaxation (1), and augmentation of smooth muscle contraction independently of the endothelium (2). The aim of this study was to explore the effects of MCD on contraction of rat femoral arteries.

Methods: Isometric force was measured in rat femoral artery in a resistance artery myograph. Arteries were stimulated to contract with a solution containing 20 mM K+ and 100 mM BayK 8644 (20K/BayK). Data are expressed as mean ± SEM, compared by Student’s t-test.

Results: Incubation of arteries with MCD (5 mM, 60 min) increased force in response to 20K/BayK from 11.84 ± 1.30 mN to 18.25 ± 2 mN (n = 12, P < 0.01). In contrast, contraction induced by 80 mM K+ was not altered by MCD (33.40 ± 1.43 mN to 33.16 ± 2.02 mN, n = 12, P = n.s.). Filipin, which also disrupts caveolae, also significantly increased force to 20K/BayK (5.36 ± 1.66 mN to 9.36 ± 2.21 mN, n = 14, P = 0.01). Cholesterol saturated MCD (Ch-MCD, 5 mM, 50 min) had no effect on its own on 20K/BayK induced force (3.15 ± 0.45 mN to 2.98 ± 0.59 mN, n = 4, P = n.s.), but reversed the effects of MCD (3.50 ± 0.62 mN in 20K/BayK, 4.93 ± 1.35 mN after MCD, 2.74 ± 0.59 mN after Ch-MCD, n=16). Functional effects of caveolar disruption have been reported to be both endothelium-dependent and endothelium-independent (1,2). Mechanical removal of endothelial cells from femoral artery reduced the ability of MCD to enhance contractions to 20K/BayK (6.62 ± 0.92 mN to 7.52 ± 1.03 mN after MCD, n = 22, P = n.s.). This result suggests MCD acts via altering basal release of an endothelial-derived vasodilator or vasoconstrictor. When endothelial release of nitric oxide (NO) was blocked by pre-incubation of arteries with L-NAME (250 μM), contraction of arteries to 20K/BayK was enhanced (6.82 ± 1.61 mN to 14.90 ± 2.67 mN, n = 6, P <0.01). Subsequent treatment of these arteries with MCD caused contraction, and L-NAME no longer had a significant effect after MCD treatment (17.74 ± 3.46 mN after MCD, 16.10 ± 3.19 mN in L-NAME and MCD, n = 6, P = n.s).


Arterial acetylation of histones is increased by a broad class II/II lysine deacetylase (KDAC) inhibitor but not by a specific HDAC8 antagonist

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Acetylation is increasingly recognised as a post-translational modification with a wide array of potential protein targets to rival that of phosphorylation. Originally associated with the modification of nuclear histones it is becoming apparent that many non-nuclear proteins are targets for specific lysine residue acetylation by lysine acetyltransferases (KATs formerly known as HATs) and lysine deacetylases (KDACs formerly known as HDACs) (1,2). Indeed, class I KDACs (1, 2, 3, 8 and 11) have been suggested to be localised in the nucleus yet in cultured vascular smooth muscle cells KDAC8 was found to bind to alpha smooth muscle actin and determine cell motility (3). The aim of this study, therefore, was to investigate in intact arterial smooth muscle the effect of a class II KDAC inhibitor, trichostatin A (TSA), in comparison to that of a specific KDAC8 inhibitor (Compound 2), on acetylation of nuclear histones. Aorta tissue isolated from Wistar rats, or rat A7r5 cells to enable a comparison between differentiated smooth muscle tissue and cultured cells, were incubated in DMEM with either TSA (3.3 μM in ethanol), Compound 2 (200 μM in DMSO) or appropriate control for 0, 1, 3 or 24 hours. Protein homogenates were analysed by western blotting for histone acetylation using

Poster Communications

PC352

PC353
an anti-acetylated H3 antibody. Densitometry data were analysed with one-way ANOVA and Bonferroni post-hoc tests, values are means ± sem. TSA induced significant (P<0.05) elevation of histone acetylation at all timepoints in both aorta tissue and cells. For example, aorta tissue: control 0 hr, 3.68±2.50 denitometric a.u., TSA 13.90±2.52 a.u., 20.6±2.52 a.u. and 27.3±2.42 a.u. at 1, 3 and 24 hours respectively (n=3). In contrast, treatment with Compound 2 was without effect on histone acetylation even though it was efficacious in inhibiting KDAC8 activity in an in vitro fluorimetric assay. Vehicle controls had no effect. Immunofluorescent staining of aorta tissue clarified a predominantly nuclear localisation of KDAC1 yet an almost entirely non-nuclear expression pattern of KDAC8. These data support the notion that KDAC8, in location and function, is distinct from other class I KDACs and is therefore unlikely to directly influence gene expression by chromatin modification. The non-nuclear protein target(s) of KDAC8 in arterial smooth muscle, and the functional consequences of such interactions, remain to be determined.

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Results: Quantitative PCR revealed relative abundance of these genes as: Best 3 approximately equal to TMEM16A >>TMEM16B (n=3). Similar expression was observed in mouse portal vein and carotid artery. In addition, all blood vessels studied expressed a Best 3 splice variant that appeared to be specific to the vasculature (n=3). Application of ZnCl2 (100 μM) had no effect on methoxamine-induced contractions. In contrast, the T16inh-01 relaxed methoxamine-induced contractions effectively (67 ± 9% relaxation at 10 μM (n=4) although the effects were relatively slow (time to maximum relaxation = 18 ± 4 min). Tannic acid had variable effects on methoxamine contractions either producing a rapidly relaxation of ~75% within 5 mins or having no effect at all.

Conclusion: TMEM16A, but not Best 3, appears to contribute to β-adrenoceptor-induced contractions.


This work was supported by the British Heart Foundation.

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PC354

Comparison of TMEM16A and Best 3 expression and function in murine thoracic aorta

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Introduction: Opening of Ca2+-activated Cl- channels (CACC) produces smooth muscle depolarization and increases contractility. Yet, the functional impact of these channels has always been difficult to determine because of the relatively ineffective pharmacological agents. However, since the original identification in 2008 of TMEM16A as a strong candidate for the molecular correlate of CACCs, there has been renewed interest in deciphering the functional role of these channels in a variety of cell types (see reviews Galietta, 2009; Kunzelmann et al., 2009). Moreover, an earlier CACC candidate, Bestrophin 3 (Best 3), has been shown to be important for cGMP-dependent Ca2+-activated Cl- conductance in rat mesenteric arteries (Matchkov et al., 2008).

The aim of this study was to ascertain the relative abundance of Best 3 and TMEM16A in murine thoracic aorta and to determine the effectiveness of two novel TMEM16A inhibitors, tannic acid and T16inh-01 (Namkung et al., 2010 & 2011), as well as the cGMP blocker ZnCl2 on β-adrenoceptor-induced contractions of mouse thoracic aorta.

Methods: Female BALB/c mice (6-8 week) were killed by overdose of pentobarbitone in accordance with Schedule 1 of the Animal (Scientific Procedures) Act 1986. Vessels were dissected and utilised in either mRNA analysis or isometric tension studies.
returned after several minutes. ICCIM responded to EFS (0.5Hz or 2Hz) with an increase in baseline Ca2+ or an increase in amplitude and duration of Ca2+ transients compared with pre-stimulation events. Similar results were obtained in 4 representative experiments. Spontaneous Ca2+-transients in cells from a group of ICCILP (within a field of view) appeared to be synchronized by 0.5Hz and 2Hz EFS with several cells firing within 2.35±1.13s (n=3) and 1.78±0.66s (n=5) of each other, respectively. Prior to stimulation, there was little apparent synchronization of events. ICCILP also responded to 10Hz with a large, prolonged rise in intracellular Ca2+, which occurred after a delay of 3.16 ± 0.47s (n=15) from the onset of EFS. These results demonstrate that bladder ICC subpopulations respond to EFS and are thus likely to be functionally innervated. The synchronicity of ICCILP following EFS suggests that they could act as a functional network, and serve as targets for parasympathetic neurotransmission, which may be integral in regulating activity within the bladder.


Financial support from INComb European Union, FP7 is acknowledged.

S Gray is supported by a BBSRC/Pfizer CASE award.

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uterine strips. ICs stained with vimentin and they occupied 1.3±0.2% of the intrabundle mass (smooth muscle cells, SMCs, stained with SM actin) NIL and this increased to 2.4±0.3% (n=6). Isolated SMCs and ICs from 20 women (NIL) were characterized. SMCs had a robust L-type voltage-gated Ca\(^{2+}\) current, which was all but absent from ICs. Large outward currents occurred in SMCs (904±180 pA at +30 mV) but small in ICs (58±10 pA). These currents were reduced by Iberiotoxin to 27±5% in SMCs but only to 85±11% in ICs, indicating a large-conductance, Ca\(^{2+}\)-activated K\(^{+}\) current (BKCa). Single cell PCR of these characterized cells revealed the presence of KCNMA1, the gene product for BKCa channels, in all 20 SMCs, but was absent in all 20 ICs. In 17 of a further 20 ICs (85%) patch-clamp recording were maintained for greater than 30 min. Spontaneous inward currents occurred irregularly with a frequency of 1 per 10±2 min. This current had a duration of 31±4 sec, amplitude of 687±146 pA, reversal potential of −9±2 mV, and PNa/PPK of 0.85 in normal patch and external solutions (n=17). Replacing KCl in the patch pipette with K-gluconate had no effect on the inward current (n=7). Reversal potential was shifted to −36±6 mV (n=6) when external Na\(^{+}\) was reduced from 143 to 40 mM. In intact strips, bursts of action potentials accompanied by contractions occurred spontaneously at a frequency of 1 per 12±3 min. The spontaneous depolarizing currents and the lack of BKCa currents would facilitate a pacemaking role for ICs. The ICs had little or no voltage-gated Ca\(^{2+}\), and never contracted. Irregular oscillations in membrane potential have previously been described in ICs under zero current conditions (1) and we now show that these are underpinned by a cation current.


Lang RJ, Hashitani H, Tonta MA, Suzuki H & Parkington HC. (2007). Role of Ca\(^{2+}\) entry and Ca\(^{2+}\) stores in atypical smooth muscle cell (SMC) phenotype. Large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (BKCa) control excitation-contraction coupling and predominate in SMCs with a contractile phenotype. Intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (IKCa) regulate proliferation during vasculogenesis and are expressed in SMCs with an immature or synthetic phenotype. We previously demonstrated fetoplacental arterial SMCs have expression of phosphatidylcholine-specific phospholipase C (PC-PLC) in this phenomenon (Witzenrath et al., 2006; Cheng et al., 2006). PC-PLC hydrolyzes phosphatidylcholine to phosphocholine and diacylglycerol which activates conventional and novel isoforms of protein kinase C, and certain non-selective ion channels, notably TRPC6. The aim of this study was to test the hypothesis that PC-PLC activation during hypoxia contributes to development of HPV. Contractile force was measured using the wire myograph technique in rat small intrapulmonary arteries (IPA) preconstricted with U46619 (20-220 nM), or in the absence of preconstriction. In vivo experiments were performed on rats with retrograde catheterization of the right ventricle and the left common carotid artery for systolic right ventricular pressure (RVP) and arterial pressure recording. Animals were anesthetized with chloralose/urethane (i.p.; 80 and 800 mg/kg respectively). Hypoxic hypoxia in vivo was achieved by mechanical ventilation with a gas mixture containing 10% O\(_{2}\) in N\(_{2}\). Hypoxia induced a biphasic contraction in preconstricted IPA. The transient phase (phase I) amounted to 44.2±6.0% of the contraction elicited by 80 mM K\(^+\) (TK) (n=7), and the sustained phase (phase II) reached 16.4±5.6% of TK. Inhibition of PC-PLC with D609 (30 μM) diminished phase I by 67% (to 14.6±1.8% TK, p<0.01) and abolished the sustained phase (-6.6±0.6% TK, p<0.01). Hypoxia induced a small but significant contraction in IPA in the absence of preconstriction, though the phases were not clearly separable (5.0±6.6% TK, p<0.01, n=7). D609 suppressed HPV in the absence of preconstriction by 60% (2.0±0.7% TK, p<0.01). Conversely, inhibition of phosphatidylinositol-specific phospholipase C with U-73122 (3 μM) did not affect HPV in IPA in the absence of preconstriction (p>0.05, n=6). Hypoxia also provoked a biphasic increase in RVP in experiments in vivo, with maximum RVP rising from 29.7±1.1 mmHg to a transient of 41.7±1.2 mmHg and a sustained value of 39.2±1.3 mmHg (p<0.01 for both phases, n=10). Intravenous injection of D609 (5 mg/kg) 30 min before hypoxia prevented the development of the pulmonary hypertension (33.1±1.8 mmHg, p>0.05, n=10). D609 did not affect changes in systemic blood pressure in hypoxia. In conclusion, the results suggest that PC-PLC may be of importance in the development of both phases of HPV, but with a greater role in sustained HPV.


This study was supported by The Physiological Society International Junior Research Grant to Strielkov I.V., 2009-2010.

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Evidence of phosphatidylcholine-specific phospholipase C involvement in hypoxic pulmonary vasocostriction

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The intrinsic mechanisms underlying hypoxic pulmonary vasoconstriction (HPV) are still poorly understood. Data obtained to date indirectly point towards the possible involvement of phosphatidylcholine-specific phospholipase C (PC-PLC) in this process.
a mixed phenotype and express both contractile and synthetic proteins. Here we test the hypothesis that SMCs isolated from fetoplacental arteries express BKCa and IKCa channels.

SMCs were isolated from chorionic plate arteries of normal term placentas using methods reported previously. Whole-cell patch clamp was used to assess K+ currents (5 mM [K+]o, 140 mM [K+]i, 0.5 mM EGTA, 0 mM [ATP]). Cells were clamped at -60 mV and depolarised from -70 mV to +80 mV for 500 ms to record current-voltage relationships. K+ channel function was assessed by extracellular application of TEA (BKCa inhibitor; 5 mM), charybdotoxin (ChTx; BKCa and IKCa inhibitor 100 nM), and ibeberiotoxin (lbTx; BKCa inhibitor; 100 nM), TRAM-34 (IKCa inhibitor; 10 μM), and 1-EBIO (IKCa opener; 100 μM). BKCa and IKCa protein expression was assessed in isolated SMCs with immunocytochemistry.

Whole-cell K+ currents (I(K)) were outwardly-rectifying and displayed spontaneous outward currents (SOCs) at depolarised potentials greater than +50 mV (n=56 cells, N=25 placentas). SOCs were abolished by TEA (n=11, N=6), ChTx (n=4, N=2) and lbTx (n=6, N=3). Outward currents were insensitive to TRAM-34 (n=3, N=2). Addition of 1-EBIO significantly increased I(K) at depolarised potentials greater than +40 mV, with a 431±62% (mean±SE) increase observed at +80 mV (pA/pF; control 6.6±2.1; 1-EBIO 29.1±6.9; P<0.05, n=22, N=10; Wilcoxon signed rank test). 1-EBIO-sensitive current (pA/pF) was inhibited by TRAM-34 (1-EBIO 34.5±10.0; 1-EBIO+TRAM-34 7.1±2.7; P<0.05; n=7, N=4), but not ibeberiotoxin (1-EBIO 15.2±11.2; 1-EBIO+lbTx 14.2±6.9; P>0.05 n=3, N=3). All isolated SMCs (N=3) displayed positive immunostaining for BKCa and IKCa.

Outward whole-cell K+ currents in chorionic plate arterial SMCs are mediated by BKCa, IKCa, and not contributed to I(K) under resting conditions; however, 1-EBIO stimulates a large increase in current which is abolished following inhibition of IKCa with TRAM-34. Therefore, fetoplacental arterial SMCs express BKCa and IKCa currents. This, coupled with the expression of BKCa and IKCa protein in SMCs, supports our previous observation that these cells have both contractile and synthetic characteristics. This mixed phenotype may be related to the dual function of fetoplacental SMCs to control both vascular resistance and promote vasculogenesis during pregnancy.


Supported by the Medical Research Council and the Manchester Biomedical Research Centre.

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Effect of modulators of the nitric oxide/cGMP pathway on L-type Ca2+ current in rabbit corpus cavernosum smooth muscle cells

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Although it is well known that nitric oxide (NO) induces erection by inhibiting tone in corpus cavernosum smooth muscle cells (CCSMC), the mode of action is not yet fully elucidated.

It has been suggested that an important contributory factor is inhibition of L-type Ca2+ current, although this has never been directly tested in CCSMC. The purpose the present study was to examine the effect of an NO donor and several modulators of the NO/cGMP pathway on L-type Ca2+ current in rabbit CCSMC. Male rabbits were humanely killed and their penises removed. The corpus cavernosum was cut into 1 mm3 pieces and CCSMC isolated using a collagenase/protease mixture. Cells were superfused with physiological saline at 37°C and studied using the perforated patch voltage clamp technique. YC-1 (30 μM), an activator of soluble guanylate cyclase, reversibly reduced the L-type current evoked by stepping from -60 to 0 mV (from -129±40 pA to -79±23 pA, p<0.05, paired t test, n=8). This effect was completely blocked by ODQ (30 μM), an inhibitor of soluble guanylate cyclase (-132±31 in ODQ and -125±29 in ODQ + YC-1, n.s., n=8). Similar results were obtained with a NO donor, DEA-NO (30 μM; current reduced from -104±14 pA to -80±13 pA, p<0.05, n=9) and 8-bromo cGMP (1 mM; from -83±21 pA to -70±22 pA, p<0.05, n=6). When the effect of sildenafil (1 μM) was examined it was found to produce only a small reduction in the current (from -132±28 pA to -116±25 pA, p<0.05, n=7) and even at the supratherapeutic concentration of 10 μM the effect was modest (reduced from -160±32 pA to -123±24 pA, p<0.05, n=5). Indeed the effects of the all of the above compounds on L-type Ca2+ current were modest, compared to their more potent effects on blocking intracellular Ca2+ waves, in CCSMC, suggesting that the latter mechanism may be more important. For comparison, we examined the effect of phentepheline (PE, 10 μM), a potent tone-inducing agonist in the corpus cavernosum. Similar to the above agents, PE caused a small reduction in L-type Ca2+ current (from -197±35 pA to -123±21 pA, p<0.05, n=8). However, despite their similar actions on L-type Ca2+ current, PE and YC-1 had markedly contrasting effects on spontaneous depolarisations (SD) in CCSMC studied in current clamp recordings. SDs have been previously shown to be mediated by Ca2+ waves activating calcium-activated Cl− currents in these cells. PE (10 μM) increased the frequency of SDs from 7.4±0.5 min−1 to 8.2±0.8 min−1 (p<0.05, n=5), while YC-1 (30 μM) reduced their frequency from 14.0±2.4 min−1 to 1.5±0.6 min−1 (p<0.05, n=4). In conclusions, these results suggest that inhibition of L-type Ca2+ current plays only a minor role, if any, in mediating the inhibitory effects of the NO/cGMP pathway on CCSMC. Christoph GJ & Hodges S. (2006). Molecular mechanisms of detrusor and corporal myocyte contraction: identifying targets for pharmacotherapy of bladder and erectile dysfunction. Br. J. Pharmacol. 147, S41–S55.

Funded by Science Foundation Ireland 07/RFP/BIMF377

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Effect of modulators of the nitric oxide/cGMP pathway on L-type Ca2+ current in rabbit corpus cavernosum smooth muscle cells

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Although it is well known that nitric oxide (NO) induces erection by inhibiting tone in corpus cavernosum smooth muscle cells (CCSMC), the mode of action is not yet fully elucidated.
Caveolae subpopulations in rat aortic smooth muscle

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Caveolins act as coat proteins for caveolae, small flask-shaped pits that form in the plasma membrane of most cells. Aside from roles in caveolae formation, caveolins recruit, retain and regulate many caveolae-associated signalling molecules and are thus central to the function of these subcellular signalling compartments (1). Of the three isoforms, caveolin-1 and -2 form hetero-oligomers in most cell types whereas caveolin-3 forms homo-oligomers in striated muscle. The ability of caveolin-3 to interact with either caveolin-1 or -2 is controversial (1). Smooth muscle cells express all three isoforms and here we use immunogold electron microscopy and coimmunoprecipitation to investigate the caveolin profile of individual aortic caveolae. To assess the subcellular distribution of caveolin-1 and -3, plasma membrane sheets were ripped off rat aortic myocytes (2-4 days in culture) onto copper grids (2), dual-labelled with isoform-specific primary antibodies against caveolin-1 and -3 and visualised by addition of gold-conjugated secondary antibodies (gold particles of 5 nm and 15 nm respectively). No labelling was observed with secondary antibodies alone, and no cross-reactivity was seen between primary or secondary antibodies. Morphologically identifiable individual caveola labelled for both caveolin-1 and -3. Particle distributions were quantified by measuring the distance between gold particles corresponding to caveolin-3 and those corresponding to caveolin-1 using ImageJ (NIH, USA) software (3). Approximately 50% of caveolin-3 particles were found within 100 nm of a caveolin-1 particle (51.7 ± 11.3%; mean ± SEM, 299 measurements over 6 separate images) compared to only 4.7 ± 3.8% of particles in simulated random distributions at equivalent density (199 measurements over 6 images, p<0.01, Student’s t test). Since caveolae are ~100 nm in diameter, this suggests that caveolin-1 and -3 coexist within a single caveola. Interestingly, some caveolae labelled exclusively for caveolin-1 (49.6 ± 12.4%; 6 images) or -3 (10.8 ± 5.3%; 6 images), indicating that caveolar subpopulations may exist containing distinct isoforms. In further experiments, antibodies against caveolin-1 coimmunoprecipitated both caveolin-2 and -3 from rat aortic homogenates, suggesting that caveolin-1 interacts with caveolin-2 and -3 in vivo (n=3). Similarly, antibodies against caveolin-3 coimmunoprecipitated both caveolin-1 and -2 (n=3). Our findings suggest that caveolin-1 and -3 form hetero-oligomeric complexes in aortic smooth muscle, but do not exclude the existence of subpopulations of caveolae containing either caveolin-1 or -3. This is consistent with studies that indicate that a proportion of smooth muscle caveolae are resistant to caveolin-1 gene ablation (4), and may mean that individual cells have multiple species of caveolae each containing distinct complements of signalling molecules.


Supported by the BBSRC and BHF

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Development and plasticity of cortical motion processing

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The macaque cerebral cortex contains a set of areas that seem to be specialised to process information about visual motion. Prime among these is area MT (V5), where neurons are exquisitely sensitive to visual motion. Signals transmitted by MT neurons have been causally linked to behavioral motion sensitivity.

We studied the development of visual motion sensitivity using electrophysiological methods in pigtail macaque monkeys by measuring the animals' sensitivity to coherent visual motion imbedded in visually-noisy dynamic random dot displays. We also measured motion sensitivity in adult macaques made amblyopic by rearing either through optical defocus or surgical deviation of one eye, using methods detailed by El-Shamayleh et al. (2010). We studied neuronal responses to visual motion in the same and other acutely prepared normal and amblyopic macaques anesthetized with sufentanil (4-20 μg/kg/h, i.v.), paralysed with vecuronium (0.1 mg/ml/h) and artificially ventilated. Anesthesia and physiological status were monitored as detailed by Movshon et al. (2005) and El-Shamayleh et al (2010).

Behavioural motion sensitivity develops over a relatively long time course, extending well past the first year of life. During this time, coherence sensitivity increases, particularly to slowing-moving stimuli. Amblyopia alters the course of development, reducing animals' sensitivity to motion viewed through the amblyopic eye, both overall and selectively at low speeds, and produces a specific deficit in the temporal integration of motion signals. Physiological measurements in MT show that neuronal responses mature in sensitivity and selectivity over a relatively shorter time course than behaviour, suggesting that the early phase of motion sensitivity development may depend on neuronal signals in MT but that later development may not. Amblyopia affects the motion sensitivity of MT neurons with a pattern that in most respects resembles the change seen behaviourally, but some features of ambylopic motion vision are explained by changes in MT and seem to depend on downstream processing.

The pattern of development and plasticity of neurons in MT suggest that these neurons occupy a central place in a set of motion sensitive mechanisms. The data suggest that the process of visual development involves not only the well known changes that take place in primary visual cortex, but also engages a cascade of developmental processes in downstream cortical areas.


The work described in this paper was done in collaboration with Yasmine El-Shamayleh, Michael Hawken, Lynne Kiorpes, Adam Kohn, Nicole Rust, and Chao Tang, and was supported by grants from NIH.

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Building the embryonic brain: the importance of intercellular communication for correct axonal wiring

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During the development of any biological structure, cells communicate with each other and, at each stage, existing cells guide the maturation of new additions. For every developing cell an extracellular source of information is vital to modulate the cell's own intrinsic processes. One of the main aims of my own research over the past 30 years has been to understand how cell-cell interactions regulate development of the mammalian brain. In this presentation I will describe some examples of this research, particularly those inspired by Colin Blakemore and his group.

Over the last 25 years our work has focussed mainly on the development of the embryonic brain, using the mouse as the model species. One of our main questions has been: how do axons from the developing thalamus navigate their complex route through the subpallium to reach appropriate target cells in the cerebral cortex? It is clear that thalamic axons do not possess detailed intrinsic information that is sufficient for them to navigate along the tortuous path to the cortex. Instead, they rely on cues from the environment that they grow into, but what are those cues?

A guidewire is a thin flexible wire inserted into a tortuous space to act as a guide for insertion of subsequent bulkier material. By analogy, in the developing brain it has long been hypothesised by researchers including Colin Blakemore, Zoltan Molnar, Christine Metin, Dennis O'Leary and others that the mechanisms by which thalamic axons attain the cortex include guidance by previously-generated guide-axons, for example reciprocal projections from intermediate structures, or from the cortex itself, back towards the thalamus. Evidence supporting this idea has come largely from observations in vivo and in vitro experiments. Here I shall present in vivo evidence from conditional mouse mutants providing further support for the importance of the descending corticothalamic axons in enabling ascending thalamic axons to cross the pallial-subpallial boundary into the cortex. In addition, I shall show that projections from prethalamic to thalamus are important for the fine organisation of thalamic axons emerging from the thalamus. The findings emphasize the importance of interactions between reciprocal axon projections in the development of brain connections.

Another source of guidance for thalamic axons as they steer towards the cortex might be diffusible molecules that attract them towards the cortex and/or repel them from inappropriate areas. In fact, our work has shown remarkably little evidence that diffusible molecules from the cortex attract thalamic axons, although the cortex does release chemotrophic molecules that stimulate the overall growth of thalamic axons. There is better evidence for molecules that repel thalamic axons from inappropriate regions as they cross towards the cortex.

Overall, the results strengthen the notion that thalamocortical pathfinding involves numerous guidance cues operating at a series of steps along the route. They highlight the critical importance of cell-cell interactions in wiring the embryonic brain.
Innate and environmental factors in the development of the visual cortex revisited

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It is more than 40 years since Colin Blakemore published his first paper on the postnatal development of the visual cortex, demonstrating the effects of rearing cats in an environment consisting of stripes of a single orientation [1]. This was followed by a series of influential papers in the Journal of Physiology, introducing first the paradigm of reverse lid suture and then examining in detail the factors governing visual cortical development and the conditions required for the maintenance of binocularity. Since then, the primary visual cortex (V1) has become a model system for studies of plasticity and learning processes in the brain. We have built on Colin Blakemore’s work along two main lines of enquiry.

1) Using optical imaging of intrinsic signals, we have provided conclusive evidence not only for the fact that stripe rearing biases the representation of orientations in V1 towards the experienced one but also for experience playing an instructive role in this process. Neurons whose innate orientation preference does not match that present in the environment shift their preference rather than lose responsiveness [2].

2) We have examined in detail the conditions under which recovery from monocular deprivation and the accompanying amblyopia can be elicited, and we have investigated what amount of daily eye patching can be imposed without incurring either significant physiological changes or amblyopia. We found that recovery from monocular deprivation both in terms of V1 physiology and behaviourally assessed visual acuity depends critically on concordant binocular visual experience. These findings demonstrate that binocular interactions are not exclusively competitive but can also be cooperative [3]. The deleterious effects of monocular eye closure can be prevented almost entirely if patching is combined on a daily basis with about two hours of binocular visual exposure [4, 5]. Under these conditions, only stereopsis and the underlying cortical disparity selectivity appear to be adversely affected to a variable extent [6]. These results have important implications for the treatment of amblyopia in children.


Identifying components of the neural wiring of the visual cortex

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Blakemore and Campbell (1969) showed, psychophysically, that there were spatial frequency and orientation tuned mechanisms in human vision. Some neurons’ receptive fields in primary visual cortex are selective for orientation and spatial frequency and are hypothesized to underlie the psychophysically defined channels. We have measured receptive field properties – including orientation and direction selectivity, spatial and temporal tuning, contrast sensitivity, selectivity for colour and extra-classical receptive field suppression – from a large population of V1 neurons within individual cortical layers. Multidimensional analysis of tuning properties within individual layers uncovers different subpopulations, both within and between layers. Additional functional and anatomical studies have been undertaken to further elucidate the wiring within the local circuits that is related to the tuning properties and response modulation of the different subpopulations. The results provide the fabric for the development of multidimensional models of receptive field properties within the cortical circuit, thereby extending the connection between psychophysically defined channels and the underlying cortical wiring and circuits.


Supported by NIH grants EY08300, EY17945

The origins of cardiac stem cells

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Candidate cells for the repair of the myocardium include mature cardiomyocytes and stem/progenitor cells. The former have been shown to be involved in regeneration of zebrafish and neonatal mouse hearts after damage or loss, but in neither case can a role for stem/progenitor cells be excluded. Furthermore, the ability of cardiomyocytes to re-enter the cell cycle, a necessary prerequisite for their involvement in regeneration, appears to be substantially diminished in the adult mammalian heart. A range of markers and methods have been used to identify stem/progenitor cells in the adult heart and some of these have been linked to cells seen during embryonic development. An attractive property of such cells would be multi-potentiality such that the endothelial and smooth muscle cells of the heart could be repaired or replaced at the same time as the myocardium. This property has been attribu-

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uted to a subset of the candidate stem/progenitor cells reported to date.

We have been studying the development of the heart using zebrafish and the amphibian, Xenopus, as models, and have discovered that the cardiac programme is closely related to the blood and endothelial programmes early in development. Indeed we have shown that they share master regulators but then that they become differentiated by virtue of FGF signalling which sets up cross-antagonistic interactions between transcriptional regulators specific to one or other tissue. We have proposed that, by extending the domain of influence of FGF signalling during evolution, the population of blood and endothelial precursors found adjacent to the heart in zebrafish and Xenopus have been recruited into the heart field to generate the bigger multi-chambered heart found in amniotes including mammals. The significance of this model for stem/progenitor cells in the adult heart may lie in the evolutionary memory, of the endothelial programme for example, retained in these more recently recruited cells. An example is the cell population expressing the transcription factor, Isl1, observed in the neonatal heart. In the embryo Isl1 marks the precursors of the second heart field which may represent this more recently recruited population. We have obtained data showing that Isl1 does indeed control the blood and endothelial programmes as well as the myocardial programme in Xenopus embryos. This role for Isl1 is downstream of Wnt and BMP signalling. A better understanding of the origins of candidate stem/progenitor cells and their programming may allow their manipulation for cardiac repair in the future.

The speaker acknowledges the skill and dedication of Tessa Peterkin, Filipa Simoes, Arif Kirmizitas, David Cleaver and Maggie Walmsley, and financial support from the MRC, the BHF and the European Union CardioCell Consortium.

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SA6

Human embryonic stem cell-derived cardiomyocytes

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Human embryonic stem cells (hESC) are presently the stem cell type with the greatest proven capacity for producing phenotypically authentic cardiomyocytes, and are therefore of great interest in terms of cardiac repair, or as a model system for cardiac physiology and pathophysiology. Embryonic-like cells can also be induced by expression of "stemness" factors within somatic cells, and these induced pluripotent cells (iPSC) can be differentiated to cardiomyocytes (iPSC-CM) using similar protocols. These raise the possibility of creating cardiomyocytes which are immune matched to the subject, or which harbour a known disease-causing mutation. These raise the possibility of creating cardiomyocytes (iPSC-CM) using similar protocols. These raise the possibility of creating cardiomyocytes which are immune matched to the subject, or which harbour a known disease-causing mutation. Based on their gene expression patterns, electrophysiological, morphological and contractile properties, the majority of human embryonic stem cell-derived cardiomyocytes (hESC-CM) or iPSC-CM initially resemble human immature cardiomyocytes, but have the capacity to mature in a number of respects. We have investigated both acute and long-term responses in hESC-CM and iPSC-CM, in comparison to adult human ventricular myocytes. Matching of excitation-contraction coupling between hESC-CM and adult myocytes is of particular concern with respect to the generation of arrhythmias. Delivery of immature myocardium into the mature ventricle has the potential to promote abnormal rhythms both because the hESC-CM have spontaneous contractile activity and because any difference from adult ventricle will produce a heterogeneous substrate. hESC-CM were followed for 5 months after induction of differentiation for beating activity, and the development of excitation-contraction coupling was quantitated by determining the relative contributions to contraction through calcium entry or calcium release from intracellular stores. Transition from the immature to mature cardiac phenotype is characterised by increasing dependence on intracellular calcium. HESC-CM were challenged with either caffeine (rapid application) or thapsigargin, both of which will empty intracellular calcium stores. While hESC-CM before day 46 were insensitive to these manoeuvres, those between day 129-150 showed prolonged relaxation, slow calcium transients and frequent cessation of beating. These were indications of development of the adult phenotype, and were comparable with effects in adult human ventricular cardiomyocytes. Sensitivity to arrhythmic challenge with bile acids (in a model of obstetric cholestatis) altered over a shorter timescale, being largely absent by 71 days. In terms of pharmacological response, stimulatory beta1- and beta2AR pathways in hESC-CM were well matched to adult human ventricular myocytes even at early time points, although the absolute sensitivity to catecholamines was 5-10-fold lower. Inhibitory muscarinic and adenosine responses were also evident, and agonists for these receptors reduced both basal and catecholamine-stimulated chronotropy. Adenosine activated through A1 adenosine receptors with a sensitivity approximately 20-fold less than in adult human ventricular myocytes, but the maximum efficacy increased over 60 days in culture. Parallel increases in muscarinic receptor responses suggest greater activity of IkAch as the key developmental change. Growth and proliferation potential are important for expansion of hESC-CM after implantation. We showed that hESC-CM retain their capacity for increase in size in long-term culture (<180 days post differentiation), although proliferative activity decreases. Exposing hESC-CM to hypertrophic stimuli such as equiaxial cyclic stretch, angiotensin II and phenylephrine (PE) increased cell size and volume, percentage of hESC-CM with organised sarcomere, angiotensinogen and ANF mRNA and immunoreactive levels and cytoskeletal assembly. Changes in cell size by PE were inhibited a range of known anti-hypertrophic agents including p38-MAPK, calcineurin/FKBP and mTOR blockers. Inhibitors of ERK, JNK and CaMKII partially reduced PE-effects; PKG or GSK3 inhibitors had no effect. Modulation of kinase signalling had independent effect on proliferative capacity and cell cycle distribution of the cells, suggesting that cell growth and cell cycle progression are separable processes in hESC-CM. Apoptotic and inflammatory responses were well developed even in early hESC-CM, but innate immune signalling through key Toll receptors was undeveloped even after 4 months of differentiation. Overall, maturation is a multifaceted process, and matching of hESC-CM to the adult phenotype requires consideration of many aspects of long and short-term cellular regulation. These findings have implications for both implantation of hESC-CM/iPSC-CM and their use as a high-fidelity model system.

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Efficient cardiac regeneration post-infarction (MI) requires the replacement of lost cardiomyocytes, formation of new coronary vessels and appropriate modulation of the inflammatory response. However, the human heart has an inadequate capacity to regenerate and insight into how to stimulate repair of the heart is currently limited. Our approach utilises the paradigm of understanding cardiovascular development to inform on adult myocardial and vascular regeneration. We previously demonstrated that the actin-binding peptide Thymosin β4 (Tβ4), required for epicardium-derived coronary vasculogenesis, can recapitulate its embryonic role and activate quiescent adult murine epicardial cells (EPDCs). Following coronary artery ligation (in isoflurane-anaesthetised mice) and stimulation with Tβ4, EPDCs facilitate neovascularisation of the ischaemic adult heart, leading to improved functional recovery. Our ongoing studies seek to determine whether EPDCs, under the guidance of Tβ4, can recapitulate a further role of the embryonic epicardium in stimulating myocardial growth. Support for such a role comes from studies by others in adult zebrafish in which the epicardium is reactivated post-injury to stimulate cardiomyocyte proliferation to achieve complete myocardial regeneration. Adult EPDCs are thus emerging as resident progenitors with the potential to sustain and repair the myocardium after ischaemic damage. The ability to revive the potential of these ordinarily dormant cells lies in the identification of key stimulatory factors, such as Tβ4, either via chemical screening or by elucidating the molecular cues used in the embryo to orchestrate cardiovascular development.

This work was funded by the British Heart Foundation.

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Stem cell-based myocardial tissue engineering

SA8

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Engineered myocardium may be utilized to model heart muscle function and drug effects or find an application in tissue engineering-based myocardial repair. Recent advances in stem cell technologies have advanced the field as they allow cardiomyocyte allocation at a large scale. The preferred stem cell source for myocardial tissue engineering are embryonic stem cells, which may soon be replaced by non-embryonic pluripotent stem cells, such as induced pluripotent and parthenogenetic stem cells. The utility of pluripotent stem cells is, however, associated with substantial caveats, including the risk of teratoma formation, uncontrolled cell specification, and limited organotypic maturation. Controlling these issues will be essential to further advance stem cell-based myocardial tissue engineering. I will discuss different tissue engineering approaches and introduce concepts for the exploitation of stem cells in myocardial tissue engineering with an emphasis on controlling fate and functionality of stem cells and derivatives.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Bone marrow stem cell therapy – a clinical viewpoint

SA9

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Initial attempts to translate the results of promising basic research into novel therapies for the treatment of diseases in man often leads to disappointment as the effects seen in the pre-clinical modeling do not translate to benefits in the clinical trials. The use of autologous bone marrow derived progenitor cells to repair the heart following ischaemic injury has been suggested by some as an example of promising basic research that has failed to show similar promise when applied to man. Results of experiments in animal models of acute and chronic myocardial ischaemia suggested that delivery of autologous bone marrow derived cells to the site of injury could lead to a dramatic recovery of cardiac function with the demonstration of various mechanisms ranging from transdifferentiation of cells to the promotion of angiogenesis described. It is not surprising that these positive results (with little in the way of negative findings published) caught the attention of clinical academics given the acute need for new treatment strategies for patients with cardiovascular disease and a rapid process of translation into clinical studies occurred. Now, several years later the results of the clinical trials in which only small improvements in cardiac function have been demonstrated in response to autologous bone marrow cell therapy indicate to some that this is yet another example of a therapeutic approach identified by basic research that has been lost in translation. Before this view is widely accepted and efforts moves away from this area of research it is important to carefully consider the significance of the results of the clinical trials and importantly the relevance of the tools that have been used to measure physiologic end-points that have been used as markers of success. The ongoing ‘REGENERATE’ series of clinical trials will be used to highlight these issues and the importance of defining success in a way that is clinically relevant.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

NAADP-mediated local calcium signaling via endolysosomal two-pore channels

SA10

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Of the established Ca2+ mobilizing messengers, Nicotinic acid adenine dinucleotide phosphate (NAADP) is arguably the most intriguing. It is the most potent, often working at low nanomolar concentrations. Unlike other Ca2+ mobilizing messengers such as inositol trisphosphate (IP3) and cyclic ADP-ribose (cADPR) which release Ca2+ from the endoplasmic reticulum, NAADP mobilizes calcium from acidic stores, including lysosomes, representing a new function for this organelle. Recent
work has identified a new class of calcium release channel, the two pore channels (TPCs), as the likely targets for NAADP (1,2). These channels are endolysosomal in localization where they mediate local Ca2+ release. Three distinct roles of these NAADP-regulated channels have been identified. The first is to regulate local Ca2+ release that may play a role in endolysosomal functions including fusion and trafficking (3). The second is to trigger global Ca2+ release by recruiting Ca2+-induced Ca2+ release (CICR) channels at lysosomal-endoplasmic reticulum junctions (1,3). The third is to regulate plasma membrane excitability by the targeting of Ca2+ release from sub-plasma membrane stores to activate plasma membrane calcium-activated channels (1). In this talk, I will discuss the role of lysosome-based NAADP-mediated Ca2+ release as a widespread trigger for intracellular calcium signalling and how studies of TPCs have enhanced our understanding of this process.


Galione, A. et al. (2009). The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endolysosomal Ca2+ release channels. Pflugers Arch. 458, 869-876.


I thank the Wellcome Trust and MRC for funding the work in my laboratory. AG is also a BHF Centre of Excellence Principal Investigator.

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SA11

Caveolins in cardiovascular cells: SUMOylation as a novel regulator

P.A. Insel

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Abundant evidence indicates that caveolae, caveolin-enriched lipid raft domains in the plasma membrane, are sites that concentrate entities involved in regulation of cardiovascular cells, including myocytes, fibroblasts and endothelial cells. Endothelial nitric oxide synthase (eNOS, NOS3) is the best-studied such entity but substantial data exist for many other types of molecules, including transporters, receptors and post-receptor components involved in signal transduction. However, many questions are unanswered regarding the basis for localization and maintenance of entities within caveolar microdomains. In addition, the contribution to caveolae to pathophysiology, other than of mutated caveolins in certain muscular dystrophies, is ill-defined. Our recent evidence indicates that SUMOylation (linkage of Small Ubiquitin-like Modifier) is a previously unappreciated modification of caveolin that occurs in cardiac myocytes and influences expression of β2-adrenergceptors, but that of β1-adrenergceptors or eNOS. SUMOylation of β2-adrenergceptors alters receptor stability and turnover by catecholamines, including by catecholamines present in serum used for the growth of cells in vitro. Our results thus identify SUMOylation as contributing to the regulation of expression of certain partners and suggest that a "SUMOylation code" may help determine localization and stability of binding partners with caveolins in cardiovascular cells.

Supported by grants from NIH

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SA12

Local calcium signalling through TRPV4 channels and IP3Rs in the vascular endothelium

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Endothelial cells occupy a unique position in the vascular regulatory environment, where they play a key role in translating neurohumoral and mechanical inputs into vasomodulatory outputs. A feature that is central to the function of endothelial cells, and represents a point of convergence of endothelial cell pathways that ultimately play a role in modulating smooth muscle tone, is elevated intracellular calcium (Ca2+). Endothelial Ca2+ levels are increased through two mechanisms: influx of extracellular Ca2+ and release of Ca2+ from intracellular endoplasmic reticulum (ER) stores. Although it is clear that inositol trisphosphate (IP3) receptors (IP3Rs) mediate Ca2+ release from the ER into the cytoplasm, the pathways that mediate the entry of extracellular Ca2+—a major contributor to changes in endothelial cell Ca2+—have remained elusive. We have recently identified IP3R-mediated, stationary Ca2+ release events ("Ca2+ pulsars") at the endothelial projections to the smooth muscle (Ledoux et al., 2008). The goal of the current study was to identify and characterize Ca2+ entry pathways in native endothelium, with a focus on transient receptor potential vanilloid 4 (TRPV4) channels. We used third-order mesenteric arteries (MAs) from mice that express fluorescent Ca2+ bioensor (GCaMP2) selectively in endothelial cells (ECs). TRPV4 agonist GSK1016790A (GSK, 10 μM) caused an EC-dependent dilatation of pressurized MAs.

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In conclusion, our results indicate that TRPV4 Ca^{2+} sparklets represent calcium influx through single TRPV4 channels, and this local Ca^{2+} can cause vasodilatation through activation of EC IK channels. Supported by AHA-PHA 10POST3690006 (SKS) and NIH HL044455, HL098243, HL095488 (MTN).

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**SA13**

Endothelial cell microdomains and integrated vascular dilatation

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Heterocellular electrical coupling between cells of the arterial wall enables conducted dilatation. It is not established whether endothelial cell Ca^{2+} signals contribute to this response at the upstream sites. We used double- or triple-cannulated rat cremaster arteries to study local and conducted dilatation and endothelial cell Ca^{2+} events. Arteries were pressurized to 70 mmHg, and the endothelial cells luminally loaded with the Ca^{2+}-indicator Oregon Green 488 BAPTA-1. Arteries developed myogenic tone, and dilated to luminal perfusion of ACh, ATP, and the activator of KATP channels, levcromakalim (LVK). Each agonist evoked both local and conducted dilatation, near maximally dilating arteries 1000 μm upstream from the region of agonist luminal perfusion. In all cases, the local dilatation was associated with increases in endothelial cell Ca^{2+}. Levcromakalim increased the frequency of spontaneous endothelial cell Ca^{2+} events, without evoking the cell-wide Ca^{2+} waves observed with ACh and ATP. This increase in activity was also observed when extracellular K^+ was raised to 10 mM, suggesting that hyperpolarization per se has an effect to increase spontaneous activity in the endothelium. The Ca^{2+} events occur within discrete regions of the endothelial cells that appear to align with endothelial cell projections to smooth muscle. Increases in Ca^{2+} events were also observed upstream from the site of agonist delivery, and we suggest are secondary to hyperpolarization at these distal sites. These data suggest that the processes associated with conducted dilatation include a component of increased endothelial cell Ca^{2+} signalling in discrete domains within these cells.

Supported by British Heart Foundation and Wellcome Trust.

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**SA14**

Organization of Calcium Signaling Pathways: Coupling of Active Na^+ Transport, Calcium Signaling and Glycolysis in Cells of the Vasculature

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A wide range of studies have demonstrated that sub-sacromemal (SSL) Ca^{2+} homeostasis is tightly coupled to regionally localized Ca^{2+} stores providing unique mechanisms for regulating smooth muscle contractility. The relations between the plasma membrane and SSL Ca^{2+} stores have not been fully described, although key components such as Ca^{2+}-sensitive K^+ channels (1) and store operated Ca^{2+} channels (2) in the plasma membrane clearly are modulated by store activity in a localized fashion. The Na-Ca exchanger (NCX) is a key player in the regulation of store Ca^{2+} load, and as such its activity can modify the activity of Ca^{2+}-sensitive channels in plasma membrane (3). Moreover, the activity of the Na-K ATPase (NKA) modulates intracellular Na^+ which can alter the driving force for Ca^{2+} removal via NCX. Previous studies have demonstrated that the α2-isoform of NKA is localized in specific regions of the plasma membrane, and it is this isoform that specifically modulates NCX activity and loading of SSL Ca^{2+} stores (4). Earlier work also demonstrated that full activity of NKA is dependent on an active glycolytic pathway in vascular smooth muscle (5). The relations between Ca^{2+} store loading, the NCX / α2-NKA couple and glycolytic metabolism will be the focus of this presentation.

Supported by NIH RO1 HL63732-01.

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**SA15**

Listening to auditory cortex: neural correlates of pitch perception

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We are able to recognize and understand speech across many different speakers, voice pitches and listening conditions. However, the acoustic waveform of a sound (e.g. for example the vowel “ae”) will vary considerably depending on the individual speaker. Moreover, the ear itself will filter the sound in a location-dependent fashion, and the “ae” may be embedded in a cacophony of other, background sounds in our often cluttered acoustic environments. Because we can perceive the pitch, timbre and spatial location of a sound source independently, it seems natural to suppose that cortical processing of sounds might separate out these attributes. However, recordings made in primary and secondary cortical areas of the ferret suggest that neural encoding of pitch, timbre and location is highly interdependent [1]. Moreover, sensitivity to these sound percepts was distributed throughout the cortical fields examined.

In order to investigate whether these distributed responses might underlie pitch perception, we compared the performance of ferrets trained in a pitch discrimination task to the pitch discrimination abilities of auditory cortical neurons [2,3]. To achieve a more robust decoding of the neural responses, we developed a population neurometric analysis, with which we decoded the activity of ensembles of simultaneously recorded units. We found several parameters of the ensemble response...
Predicting perceptual decisions from the activity of individual sensory neurons: does it imply a causal role of these neurons?

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It has long been shown that during perceptual decision tasks, the activity of sensory neurons correlates with a subject’s percept, even when the physical stimulus is identical. This correlation can be used to predict a subject’s choice at the end of a trial from the activity of these sensory neurons during the trial. How well one can predict the choice depends on the strength of the neuron-behavior correlation, and is commonly quantified as a neuron’s “choice-probability”. The existence of choice-probability in sensory neurons is often seen, at least implicitly, as evidence that these sensory neurons play a causal role in the decision. A widely held view is that these neuron-behavior correlations, i.e. choice probabilities, reflect the causal effect of feed forward noise in these sensory neurons on the decision. However, choice probabilities could also result from different brain-states associated with the perceptual choice (top-down effects). In the first part of my talk, I will present evidence that challenges the feed forward causal explanation. We trained monkeys to perform a binocular disparity discrimination task. While they performed the task, we recorded the activity of disparity-selective neurons in their V2. We used white-noise analysis to measure tuning-functions of V2 neurons associated with choice and simultaneously measure how the variation in the stimulus affects the monkeys’ perceptual decisions. In causal models stronger effects of the stimulus upon decisions, mediated by sensory neurons, are associated with stronger choice-related activity. However, we find that over the timecourse of the trial, these measures change in opposite directions—at odds with causal models. An analysis of effect of reward size supports the same conclusion. Finally, choice was associated with changes in neuronal gain that are incompatible with causal models. All three results are readily explained if choice is associated with changes in neuronal gain caused by top-down phenomena that closely resemble attention. We conclude that top-down processes contribute to neuron-behavior correlations.

In the second part of my talk, I will present data suggesting that the organization of top-down signals may require a cortical map for the features relevant for the perceptual task. This would explain why studies using discrimination tasks based on binocular disparity in area V1 have not found these correlations, as V1 contains no map for binocular disparity. A prediction of this scheme is that activity of V1 neurons correlates with decisions in an orientation discrimination task. To test this prediction, we trained two macaque monkeys in a coarse orientation discrimination task of orientation bandwidth of the filter. While the trained animals performed this task, we recorded from orientation selective V1 neurons. For both monkeys we observed neuron-behavior correlations (mean choice probability 0.55). The mean choice probability in each monkey exceeded chance level (p<0.05). These results confirm the prediction from our proposed scheme. Choice probabilities are only observed for neurons that are organized in maps for the task-relevant feature. We speculate on a novel function for the cortical columnar architecture: efficient wiring of top-down signals.

Decoding the cortical computations that support 3-D vision

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Estimating the three-dimensional (3-D) structure of the environment is a principal function of the visual system, facilitating key tasks such as segmentation, object recognition, material perception and the control of movement. Despite the central importance of 3-D estimation, we still know relatively little about the functional roles of different cortical areas in processing depth. In particular, how do responses to depth signals in different cortical areas relate to our perception of 3-D shape? This presentation will review recent human brain imaging work that has sought to test the functional roles of different por-
tions of the dorsal and ventral visual streams in supporting 3-D vision. The general strategy for this fMRI work is to (a) use parametric stimulus manipulations that can be cross-referenced to psychophysical- and electrophysiological-studies; (b) use high-resolution data acquisition to obtain fMRI signals that are closer to the neural representation; (c) examine information related to depth at the level of individual voxels and patterns of voxels using advanced statistical analysis methods. I will start by describing work that addresses the representation of depth from binocular disparity. In particular, I will describe studies that have used fMRI decoding approaches to test for cortical representations of binocular disparity that are compatible with the observer’s perception of depth from disparity. This work makes use of a low-level stimulus manipulation (reversing the contrast polarity of stimuli viewed by the two eyes) to study stimuli in which depth structure is-, or is not-, clearly visible. This technique reveals disparity representations that are compatible with perceptual judgments in higher portions of the ventral stream, and intermediate and higher portions of the dorsal stream. In contrast, responses in early visual areas and intermediate ventral areas contain information that is dissociated from perception. Further, using parametric manipulations, the work demonstrates important differences between the disparity representations in higher portions of the dorsal and ventral streams. Specifically, higher dorsal areas contain information that relates to the particular disparity that is viewed by the participant; in contrast, higher ventral areas show responses that relate to disparity sign (near vs. far) rather than disparity magnitude. This suggests a difference between dorsal and ventral representations related to metric vs. configurual depth.

The second portion of the talk will address the question of how information from different depth cues (disparity and relative motion) is synthesised to support 3-D perception. Behavioural tests show humans combine depth cues near-optimally, a feat that could depend on: (i) discriminating the outputs from cue-specific mechanisms, or (ii) fusing signals into a common representation. I will describe fMRI studies that combine established psychophysical methods, high-resolution imaging and advanced analysis methods to address the processing of individual depth cues and their combination. I will describe approaches needed to identify cue fusion, and highlight different representational codes in dorsal vs. ventral cortical areas. The work suggests that area V3B/KO in the dorsal stream plays an important role in representing depth from different cues. Activity in this area may underlie improved performance when different cues are combined to represent 3-D depth.

The findings from both portions of the talk suggest perceptually-relevant 3-D representations in both dorsal and ventral visual areas. The empirical approach outlined in the talk provides a means of dissociating these representations to reveal the network of functional computations that support 3-D perception.


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Living on the edge - how evidence, reward, and electrical stimulation affect decision making

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Perceptual decisions about ambiguous visual stimuli are influenced by information about both the sensory environment and the value of alternative actions. It is not yet understood where in the neural structures underlying the decision-making process these variables are combined. Introduction of an artificial electrical stimulus into cortical visual area V5/MT of the Rhesus macaque has enabled us to investigate how sensory and value information interact to shape perceptual decisions about structure-from-motion stimuli.

Two Rhesus macaques were trained to discriminate the direction of rotation of a structure-from-motion cylinder (duration 2 sec) with an eye movement to one of two choice targets. The stimulus consisted of two transparent surfaces of random dots moving in opposite directions. The two surfaces could be presented at different relative depth, so for some decisions more sensory evidence was available; other trials were ambiguous. Correct responses were rewarded with a fluid reward. Once trained, animals were implanted under general anaesthesia with a headholder and a chamber for access to the cortex (7-8 mg/kg/hr alfaxonale and alfadalone acetate i.v. or 1-3% isoflurane to effect) (see Dodd et al 2001; Read and Cumming 2003 for Methods).

During the experiment, V5/MT cortical sites with 200-300 μm of consistent tuning for cylinder’s direction of rotation were identified. Cylinder stimuli were matched to the rotation preference at that cortical site. On 50% of the trials, while the monkey viewed the rotating cylinder, we electrically stimulated the site with a train of 20 μA biphasic pulses. The proportion of reports in one direction as a function of disparity (psychometric functions) were fitted for stimulated and non-stimulated trials with a pair of cumulative Gaussian curves, separated by a horizontal offset or shift. At 27/48 sites, the electrical microstimulation induced a significant shift in the monkey’s perceptual reports in favour of the cylinder tuning preference of the neurons at the stimulated site (Chi-square, p < 0.05).

The size of the reward available for a correct report at the end of each trial was varied systematically, such that as the number of consecutive correct choices increased, the size of the available fluid reward increased in two steps up to a maximum. At each stimulation site, trials were split into two conditions, maximal and sub-maximal expected reward size. For trials where the available reward size was maximal, the effect of electrical microstimulation was decreased (Wilcoxon sign-rank test, p < 0.001). This shows that the artificial signal that we inserted directly into sensory cortical area V5/MT was affected differently from stimulus-dependent, non-microstimulated cortical activity as the reward condition changed.

The application of a simplified drift-diffusion model of perceptual decision-making to the psychophysical data revealed that in order to explain this result, the effects of expected reward must act on the level of sensory representations prior to the proposed integration of sensory and value evidence into a decision variable in sensorimotor structures. This strongly implies that reward affects representations of sensory evidence in visual cortex, which project to sensorimotor structures.


This work was supported by the Royal Society, the Wellcome Trust, NIH and Exeter College.

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SA19

Sensory decision making in the human brain: From perception to valuation

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Perceptual decision making is the act of choosing one option or course of action from a set of alternatives based on the available sensory evidence. Thus, when we make decisions, sensory information must be interpreted and translated into behavior. Decision-making research has resulted in mathematical models of the assumed underlying cognitive processes and sequential sampling models are particularly successful in explaining response time data and accuracy in two-choice reaction time tasks. Recent studies in monkeys and humans have begun to model not only psychophysical but also neurophysiological data as a diffusion-to-barrier process providing a quantitative link between behavior (decision outcome) and neural activity (decision processing). In my talk I will discuss how we can use this framework to better understand the neu-rocognition of human decision making. Specifically, I will discuss a) results of a recent TMS study on the role of the dorsolateral prefrontal cortex in perceptual decision making, b) how human decision makers adapt thresholds to maximize reward in a perceptual decision-making task, and c) whether the mechanisms identified for perceptual decision making generalize to value-based decisions.

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SA20

Neural Control of the Circulation During Exercise

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During exercise the magnitude of the cardiovascular response is closely matched to the intensity of the exercise. In achieving this appropriate matching, an important role is played by the autonomic nervous system. Over 100 years ago Johansson (Scand. Arch. Physiol., 5:20-66, 1893) postulated that there were two possible mechanisms which regulated this response. In one mechanism the changes in autonomic nerve activity to the heart and circulation are caused by signals arising in a central area of the brain and in the other mechanism the changes in autonomic nerve activity are caused by signals arising in the contracting skeletal muscle. Studies by Krogh and Lindhard (Journal of Physiology, 47:112-136, 1913) supported the central mechanism concept which they first termed “Cortical Irradiation”. Later studies by Alam and Smirk (Journal of Physiology, 89:372-383, 1937) supported the response originating in muscle which they termed the “Blood Pressure Raising Reflex”.

In 1971 two studies were performed in Oxford which furthered our understanding of these two mechanisms. In one of these studies performed by McCloskey and Mitchell (Journal of Physiology, 226: 173-190, 1972) it was shown in cats that a reflex arising in the contracting skeletal muscle reflexly increased blood pressure and heart rate. This finding confirmed a study by Coote, Hilton and Perez-Gonzalez (Journal of Physiology, 215: 789-804, 1971) and further defined, by using anodal and anesthetic blocking techniques, that thinly myelinated (Group III or Aδ) and the unmyelinated (Group IV or C) afferent nerve fibers were responsible for the reflex changes in blood pressure and heart rate. This mechanism is now commonly called the “Exercise Pressor Reflex”.

In the second of these studies performed by Goodwin, McCloskey and Mitchell (Journal of Physiology, 226: 173-190, 1972) it was shown in humans that a central mechanism could also increase the blood pressure and heart rate during static contraction at a fixed force. In this study high frequency vibration of a tendon was used to activate the primary afferents of the muscle spindles (Ia). If these afferents are activated in a contracting muscle, they reflexly increase the motor activity of the muscle, so less central command is required to maintain the same force. However, if the (Ia) afferents are activated in the antagonist muscle, they cause a reflex inhibition of the contracting muscle, so that a greater central command is required. When the same force was achieved with less central command, the elevation in blood pressure and heart rate was decreased, and when the same force was achieved with more central command, the elevation in blood pressure and heart rate was increased. From these findings it was concluded that descending motor commands from higher brain centers had an effect on the cardiovascular control centers during exercise and this was termed “Central Command”.

During the last 40 years a much greater understanding of the neural control of the circulation during exercise has been achieved. In addition to a more detailed knowledge of the mechanisms involved in central command and the exercise pressor reflex, it has been shown that the arterial baroreceptors also play a role in determining the autonomic nervous system activity to the heart and circulation during exercise. All three of these neural mechanisms play a role in regulating the precise response of the cardiovascular system to the intensity of exercise.

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SA21

Central command: feedforward control of sympathetic and parasympathetic outflows and the arterial baroreflex during spontaneous motor activity in animals

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Both feedforward control by a signal descending from higher brain centres (termed central command) and feedback control by an afferent signal from contracting skeletal muscle (termed exercise pressor reflex) play a role in autonomic regulation of the cardiovascular system during exercise. Extensive investigations about exercise pressor reflex, conducted using
anesthetized animals and decerebrate animals, revealed the characteristics of afferent and efferent activity and reflex centres in the brain stem. However, little is known regarding central command, particularly 1) the characteristics of the centrally-induced responses in sympathetic and parasympathetic outflows, 2) an interaction between central command and arterial baroreflex, and 3) the CNS origin(s) responsible for the central command. The reason is that it is difficult to conduct a comprehensive study during voluntary or spontaneous exercise in conscious or unanaesthetized animal preparations. Recently we have conducted a series of experiments to identify the above-mentioned questions using conscious, intact animals and unanaesthetized, decerebrate animals. All surgical procedures (the decerebration, implantation of an electrode on the renal nerve for measuring renal sympathetic outflow, and insertion of catheters into the carotid artery and vein) were conducted under halothane anaesthesia. We found that renal sympathetic outflow and heart rate abruptly increased immediately before or at the onset of spontaneous body movement or locomotion in decerebrate animals as well as conscious animals. The finding led us to a framework that central command may be generated in the caudal part of the diencephalon and/or regions within the brainstem, which may be triggered by descending output from the cortical structures, because the decerebration disconnects the cerebral cortex and the rostral diencephalon from the lower brainstem. Our recent study also provided a surprising result that a mechanical component of the exercise pressor reflex is suppressed in the conscious condition but becomes evident in the anaesthetized condition with pentobarbitone and propofol or the decerebrate condition, suggesting an influence of anaesthesia and cortical output on the exercise pressor reflex. When assessing the effect of central command on the baroreflex bradycardia in response to aortic nerve stimulation given at various phases before and during exercise, we revealed that central command interacts with the arterial baroreflex and produces a selective inhibition of the cardiac component of the baroreflex, which may in turn evoke cardiac sympathetic stimulation for cardiac acceleration, rather than cardiac parasympathetic withdrawal. Finally, regarding the CNS origin(s) responsible for the central command, we would like to propose a hypothesis that the mesencephalic ventral tegmental area (VTA) may be crucial for generating central command. The reason is that it is difficult to conduct a comprehensive study during voluntary or spontaneous exercise in conscious or unanaesthetized animal preparations.

Recently we have conducted a series of experiments to identify the above-mentioned questions using conscious, intact animals and unanaesthetized, decerebrate animals. All surgical procedures (the decerebration, implantation of an electrode on the renal nerve for measuring renal sympathetic outflow, and insertion of catheters into the carotid artery and vein) were conducted under halothane anaesthesia. We found that renal sympathetic outflow and heart rate abruptly increased immediately before or at the onset of spontaneous body movement or locomotion in decerebrate animals as well as conscious animals. The finding led us to a framework that central command may be generated in the caudal part of the diencephalon and/or regions within the brainstem, which may be triggered by descending output from the cortical structures, because the decerebration disconnects the cerebral cortex and the rostral diencephalon from the lower brainstem. Our recent study also provided a surprising result that a mechanical component of the exercise pressor reflex is suppressed in the conscious condition but becomes evident in the anaesthetized condition with pentobarbitone and propofol or the decerebrate condition, suggesting an influence of anaesthesia and cortical output on the exercise pressor reflex. When assessing the effect of central command on the baroreflex bradycardia in response to aortic nerve stimulation given at various phases before and during exercise, we revealed that central command interacts with the arterial baroreflex and produces a selective inhibition of the cardiac component of the baroreflex, which may in turn evoke cardiac sympathetic stimulation for cardiac acceleration, rather than cardiac parasympathetic withdrawal. Finally, regarding the CNS origin(s) responsible for the central command, we would like to propose a hypothesis that the mesencephalic ventral tegmental area (VTA) may be crucial for generating central command. The reason is that it is difficult to conduct a comprehensive study during voluntary or spontaneous exercise in conscious or unanaesthetized animal preparations.

SA22

Identifying the neurocircuitry underpinning ‘central command’ during exercise in humans

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For almost one hundred years the neurocircuitry that underpins the cardiovascular and respiratory responses to exercise (‘central command’), and how it integrates with the muscle pressor reflex, has been sought. Animal experiments and functional imaging studies have provided clues, however, in humans the underlying electrophysiological activity of putative sites has never been measured until now. Functional neurosurgery has recently provided an opportunity to study these circuits directly. Emerging evidence suggests that the peri-aqueductal grey (PAG) could be a major site that integrates the cardiorespiratory response to exercise. 1,2 We assessed the spectral changes in local field potentials (LFPs) recorded from deep brain nuclei in patients who had undergone neurosurgery for movement or pain disorders. We focused on the PAG, and compared it to other areas that might make up the sub-cortical circuitry, i.e. the internal globus pallidus (GPI), subthalamic nucleus and thalamus. Patients with electrodes implanted were asked to perform light exercise on a cycle ergometer (study 1), or light handgrip exercise, followed by arterial occlusion of the exercised limb in order to maintain the pressor response (study 2). In the first study, we re-visited the Krogh and Lindhard paradigm of 1913. Recordings were made during anticipation of exercise, actual exercise and recovery in an attempt to dissociate the exercise command from the movement itself. Anticipation of exercise resulted in increases in heart rate, arterial blood pressure and ventilation. The greatest neural changes were found in the dorsal lateral periaqueductal grey area (PAG) where anticipation of exercise was accompanied by an increase in activity. In the sub-thalamic nucleus there was a reduction during anticipation, but an increase with exercise. No significant changes were seen in the GPI during anticipation of exercise. In study 2, we re-visited Alam and Smirk’s paradigm of 1937. Here the dorsal lateral PAG also showed significant increases in neural activity during occlusion following exercise. This period was associated with maintained elevated arterial blood pressure. Further increases in exercise intensity, and ischaemic exercise resulted in corresponding increases in PAG activity and ABP. No significant changes were seen in the activity of other nuclei during occlusion following exercise or during ischaemic exercise. When all data are viewed together, we provide direct electrophysiological evidence highlighting the PAG as an important subcortical area in the neural circuitry of the cardiovascular response to exercise, since stimulation of this structure is known to increase arterial blood pressure in conscious humans 3.


This work was undertaken in collaboration with A.L. Green S.D. Basnayake and T. Aziz. It was supported by the Oxford Biomedical Research Centre.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

SA23

Role played by the ASIC and P2X receptors in evoking the exercise pressor reflex in animals

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In anesthetized or decerebrated animals, the exercise pressor reflex is evoked by static contraction of the hind limb muscles and results in increased arterial pressure, heart rate, myocardial contractility and ventilation. The sensory arm of the reflex is comprised of group III and IV muscle afferents (McCloskey & Mitchell, 1972). The former are thinly myelinated and are thought to be primarily mechanically sensitive whereas the latter are unmyelinated and thought to be primarily metabolically sensitive (Kaufman et al., 1983). Although several metabolic by-products of contraction are thought to be involved in stimulating group IV metaboreceptors, the receptors responsible are still being investigated. Likewise, metabolic by-products of contraction have also been shown to sensitize group III afferents, and the receptors responsible for it are still under investigation.

In the experiments to be described, we have focused on the acid sensitive ion channel (ASIC), which is stimulated by lactic acid, and the purinergic (P) 2 channel, which is stimulated by ATP. Both lactic acid and ATP are well established metabolic by-products of contraction, and their concentrations have been shown to increase in the interstitium of muscle during both static contraction under freely perfused conditions and during static contraction under ischemic conditions. We have found that blockade of the P2 channel with PPADS attenuated both metabolic and the mechanical components of the exercise pressor reflex (Kindig et al., 2006; Kindig et al., 2007). Similarly, we found that blockade of the ASIC channel with either amiloride or a variety of antagonists specific to the ASIC 3 channel attenuated the metabolic component of the reflex (Hayes et al., 2008).

In addition, we have recorded the impulse activity of group III and IV afferents with endings in the triceps surae muscles while decerebrated cats were made to walk on a treadmill. A precoccicular-postmamillary decerebration was performed while the lungs were ventilated with isofluorane (5%) in oxygen. Walking was induced by electrical stimulation of the mesencephalic locomotor region, a maneuver which recruited alpha motoneurons from slow to fast as well as caused them to fire asynchronously. Both effects are known to be displayed by alpha motoneurons during dynamic exercise in intact conscious cats. We found that most group III afferents responded in synchrony with the contraction phase of the step cycle, whereas most group IV afferents responded irregularly with respect to the step cycle (Hayes et al., 2006). Moreover, the responses to dynamic exercise by the group III afferents were prevented by gadolinium, which blocks mechanogated channels, whereas the responses to exercise by the group IV afferents were not blocked by gadolinium (Hayes et al., 2009). During treadmill walking, the triceps surae muscles increased their oxygen consumption only two and half fold, a level which does not appear to be consistent with a mismatch between metabolic demand and blood/oxygen supply. This latter finding suggests that group IV afferents, although capable of responding to ischemic stimuli, may also be capable of responding to a muscle metabolite that is not generated by a mismatch between blood/oxygen demand and supply in exercising muscles.


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SA24

Human investigations into the exercise pressor reflex in health and disease

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Alam & Smirk discovered that influence from an arm and thereby, presumably, from skeletal muscles affects cardiovascular control during exercise. Physical exercise is associated with reduced muscle oxygenation and they found that post-exercise muscle ischemia maintains the exercise blood pressure rather than heart rate. In patients with Brown-Séquard’s syndrome Alam & Smirk further established that this blood pressure raising effect of muscle ischemia is by a neural mechanism. That observation is confirmed with sensory blockade and in patients with spinal cord lesions. With mild epidural anaesthesia (1% lidocaine), there is no effect on blood pressure or heart rate during cycling exercise, but the blood pressure response becomes attenuated with the use of 0.25% bupivacaine. Furthermore, during paralyzing epidural anaesthesia with 0.5% bupivacaine or 2% lidocaine, electrically evoked exercise is without a pressor response as is the case when similar exercise is carried out in patients with spinal cord injury. In these patients, the increase in heart rate during exercise is eliminated when thigh cuffs hinder that blood from the working muscles reaches the central circulation. Experiments can also be arranged to demonstrate an effect of “central command” (the central nervous system) and cardiac preload for cardiovascular regulation during exercise. Yet, it remains that the elevated blood pressure during exercise with a large muscle mass is dominated by the muscle pressor reflex.

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SA25

Human Investigations into the Arterial and Cardiopulmonary Baroreflex: Exercise

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After considerable debate and key experimental evidence, the importance of the arterial baroreflex in mediating the appropriate neural cardiovascular adjustments to exercise is now well accepted. What may be less appreciated is the influence of the cardiopulmonary baroreceptors and its potential interaction with the arterial baroreflex during physical activity. Although the majority of research in humans regarding these low pressure receptors, which are located in the heart and lungs, has focused on neurohumoral adjustments to orthostatic stress, the limited information available regarding exercise and the cardiopulmonary baroreflex provides evidence for a role in mediating sympathetic nerve and arterial blood pressure responses. Indeed, studies have indicated that the removal of the inhibitory influence of the cardiopulmonary baroreceptors facilitated the exercise pressor and sympathetic nerve activity responses during exercise. In addition, recent investigations have demonstrated an interaction between cardiopulmonary baroreceptors and the arterial baroreflex during dynamic exercise. Increasing central blood volume and loading the cardiopulmonary baroreflex reduced the magnitude of exercise-induced increases in blood pressure as well as the resetting of the carotid baroreflex. These data are consistent with previous reports and strongly suggest that changes in cardiopulmonary baroreceptor load influences carotid baroreflex resetting during dynamic exercise. Furthermore, it is likely that neural inputs from the cardiopulmonary baroreceptors play an important role in establishing the operating point of the arterial baroreflex. Overall, several sets of data will be presented demonstrating that inputs from the cardiopulmonary baroreceptors can influence arterial baroreflex resetting and the neural cardiovascular adjustments to dynamic exercise.

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SA26

Molecular mechanism and function of acid-sensing channels (ASICs)

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ASIC channels are the simplest version of ligand-gated ion channels. Their ligand is the proton. There are 3 major genes for this channel family coding for ASIC1, ASIC2 and ASIC3 channels and there are spliced versions of these channels. ASIC channels assemble into homo or heterotrimers and are permeable to Na+ and, for some of them, to Ca2+. Different subunit assemblies have different biophysical properties (activation, inactivation, reactivation, pH-dependency ...) and different physiological functions. These channels are highly expressed in the central as well as in the peripheral nervous system and beside their structure-function activity, their role will be particularly discussed in relation with pain.

Local acidification is associated with most types of pain events. The presentation will show how the ASIC3 channel, because it is capable to sense very small pH variations, hyperosmolarity and many of the factors liberated in the course of inflammation, is a key channel for a large variety of pain states (inflammatory pain, post surgical pain ...). It will also demonstrate the importance of the ASIC1a channel in the spinal chord and the spectacular effects of its blockade against all types of pain including neuropathic pain.

The presentation will describe a series of venom peptides with a potent analgesic potential acting on ASIC3 or ASIC1a channels and will explain how they interact with ASIC channels and why they might become interesting drugs.

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SA27

G protein-coupled proton receptors: Expression, characterisation & function

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Blood pH is maintained in a narrow range around pH 7.4 mainly through regulation of respiration and renal acid extrusion. The molecular mechanisms involved in pH homeostasis are not completely understood. Acid-sensing ion channels are activated at relatively low pH (below 6.5), their setpoint is different from normal plasma pH. We have identified a family of G protein-coupled receptors that have proton-sensing properties, and show halfmaximal activation at pH 7.2-7.4. These receptors are ovarian cancer G protein-coupled receptor 1 (OG1 aka GPR68), GPR4, and T cell death-associated gene 8 (TDAG8 aka GPR65). These membrane proteins had been originally described as receptors for bioactive lipids, however, these findings were later retracted. OGR1 is expressed in bone, kidney, and the vasculature. Recent data suggest that this receptor regulates acidosis-induced bone resorption, calcium secretion in the kidney, and vascular tone. GPR4 shows a rather specific expression in endothelial cells and modulates angiogenesis. TDAG8 is restricted to the immune system. Its role in the control of inflammatory processes is being explored. Pharmacological modulation of these proton-sensing GPCRs may have therapeutic potential for the treatment of immune disorders and cancer.

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SA28

Role of carbonic anhydrase IX in the control of intracellular and extracellular pH in three-dimensional tumour-cell growths

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H+ ions are highly reactive and potent modulators of cellular physiology. Out of convention, their concentration is typically expressed using a logarithmic pH scale. Cells must regulate their intracellular pH (pHi) in order to produce a milieu that is conducive for biochemical reactions, many of which work opti
nally only within a narrow pH range. However, cellular respiration can disturb pH balance by loading the cytoplasm with CO₂ and lactic acid. If the cellular removal of these products is inadequate, their intracellular spontaneous ionisation can reduce steady-state pH₄ significantly. Membrane-bound transporters, such as Na⁺/H⁺ exchange, can remove this excess of H⁺ ions. The process of acid-removal is then completed by the diffusion of extruded H⁺ ions away from the respiring cell. This is important for maintaining a constant extracellular pH (pHₑ). An alternative to trans-membrane H⁺-flux is the transport of HCO₃⁻ ions into the cell by means of proteins such as Na⁺/HCO₃⁻ cotransport. Imported HCO₃⁻ ions react with excess intracellular H⁺ ions, producing the membrane-permeant product, CO₂. Diffusion of CO₂ across the membrane and away from the cell completes the process of acid-extrusion. Regulation of pHᵢ is therefore a process involving buffering reactions, membrane transport and diffusion.

Tumours represent an unusual challenge to pHᵢ regulation because of their high metabolic rate (hence elevated acid-production), typically inadequate blood perfusion (hence weaker cell-capillary diffusive coupling) and a high proliferative rate that requires an alkaline pHᵢ. Cancer tissue has been shown to express high levels of the membrane-tethered, extracellular-facing enzyme carbonic anhydrase IX (CAIX; Pastorek et al., 1994). It has been speculated that this enzyme is important for pH regulation because it catalyses the reversible reaction CO₂ + H₂O = H⁺ + HCO₃⁻, which involves H⁺ ions and the components of CO₂/HCO₃⁻ buffer. We have studied the role of CAIX in three-dimensional, multi-cellular growths (radius 100-300 μm; made of 10,000-200,000 cells) cultured from cancer-derived cell lines, and imaged confocally for pHᵢ with carboxy-SNARF-1 or pHr with membrane-impermeant fluorescein-derivatives. Using spheroids grown from renal bladder RT112 and colon cancer HCT116 cells, we have shown that CAIX activity facilitates CO₂ venting from cells (Swietach et al., 2008, 2009). Spheroids made of cells over-expressing CAIX had smaller radial pHᵢ gradients, with notably less acidic pHᵢ at the core, compared to spheroids treated with CA inhibitors or spheroids made of sham-transfected cells. CAIX can facilitate CO₂ efflux by hydrating extracellular CO₂ and thereby maintaining a steep CO₂ efflux gradient. Using HCT116 spheroids, we have confirmed that CAIX activity lowers pHᵢ by releasing H⁺ ions extracellularly (Swietach et al., 2009). This process may contribute towards the low pHᵢ typically measured in tumours. Moreover, low pHᵢ has been proposed to underlie the selection pressure favouring the more pH₄-tolerant cancer cells over normal cells. The other product of CAIX-catalysed CO₂ hydration, HCO₃⁻, can be taken-up by cells to regulate pHᵢ. We have shown that HCT116, RT112, colon HT29, breast MDA-mb-468, cervical HeLa and pancreatic MiaPaca2 cells produce a significant HCO₃⁻-flux that regulates pHᵢ in parallel with H⁺-flux. The magnitude of HCO₃⁻-flux was less variable than that of H⁺-flux. HCO₃⁻ transport may therefore represent a constitutive element of pHᵢ regulation, important for cancer pHᵢ homeostasis (Hulikova et al., 2010). In some cell lines, notably HCT116 and MDA-mb-468, H⁺-flux was greater than HCO₃⁻-flux. However, spheroids grown from these cells were unable to regulate pHᵢ in the absence of extracellular CO₂/HCO₃⁻ buffer. Mobile buffers such as CO₂/HCO₃⁻ facilitate H⁺ ion diffusion in the unstirred extracellular space of tumours. Without this H⁺ ion chaperoning, sustained extrusion of H⁺ ions from cells would reduce pHᵢ to levels that may inhibit membrane-bound pHᵢ regulators. To maximise the H⁺ ion shunting capacity of CO₂/HCO₃⁻ buffer, the protonation and deprotonation kinetics must be catalysed by CA activity. We have shown that H⁺-flux in MDA-mb-468 spheroids was reduced significantly in the presence of CA inhibitors.

In summary, CAIX serves an important role in tumour pHᵢ regulation by (i) facilitating trans-membrane CO₂ efflux, (ii) supplying substrate for HCO₃⁻-dependent pHᵢ regulating proteins, and (iii) facilitating extracellular H⁺ ion mobility. Inhibition of CAIX activity may disrupt the powerful pHᵢ regulatory apparatus in cancer and provide a novel target for therapeutic interventions.


This work has been supported by the Medical Research Council, Royal Society and British Heart Foundation.

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**Proton translocation by the voltage-gated proton channel Hv1: insights from molecular simulations**

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The Hv1 voltage-gated proton channel enables rapid, gated proton flux across membranes. Such proton flux may occur via a Grothuss proton ‘hopping’ mechanism as in a number of other membrane proteins and model systems. The fold of the Hv1 channel transmembrane domain is homologous to that of the voltage-sensor domain found in voltage-gated potassium, sodium, and calcium channels. This domain consists of four transmembrane helices (S1 to S4), of which S4 contains multiple positively charged arginine residues implicated in voltage sensing. We have used molecular modelling and simulation studies to explore structure/function relationships in the Hv1 channel. In particular we focus on the nature of a central water-filled pore in relation to the likely proton pathway, and the interactions of the Hv1 transmembrane domain with the surrounding lipid bilayer environment. Homology modelling of the Hv1 pore domain used the X-ray structures of the KvAP (pdb id 1ORS) and Kv1.2/2.1 chimera (pdb id 3LNM) potassium channel voltage-sensor domains as templates. Coarse-grained molecular dynamics simulations were used to predict the location of Hv1 within a phospholipid bilayer. More detailed analysis of these simulations revealed local lipid interactions with and perturbation of the bilayer by the inserted Hv1 protein (1). To gain structural insights into the proton permeation pathway in Hv1, we conducted atomistic MD simulations and analysed the extent of penetration of water into the Hv1 channel. As the template X-ray structures are thought to correspond to the ‘up’ state of the Kv voltage sensor (i.e. the state in a depolarised membrane) it is likely that our Hv1 model represents an open state of the channel. The distribution of water molecules in the simulations reveals a continuous column of
waters in the central Hv1 crevice (see Figure 1). Significantly, such continuous water columns were not observed in simulations of other voltage-sensor domains (KvAP, Kv1.2/2.1, Kv1.2) which do not support proton transfer. Neutralizing mutagenesis of candidate ionizable residues lining the aqueous crevice that could support Grotthuss H+ transfer failed to abrogate the proton conductance. We therefore conclude that the internal water wire is the most likely pathway for proton transfer through Hv1 (2). The importance of intraprotein water molecules for mediating H+ transfer may have relevance for understanding voltage-sensor channelopathies such as hypokalemic periodic paralysis.

Figure 1.
Snapshot from a simulation of Hv1, showing the central column of water. The lipid bilayer is omitted for clarity.


This work was supported by grants from the Wellcome Trust and the BBSRC.

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Proton-coupled Ca2+ signalling in heart: role of Na+-H+ exchange, mitochondria, and the L-type Ca2+ channel
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Ca2+ signalling in heart displays a complex and dynamic sensitivity to pH. H+ ions are end products of metabolism and must be removed if cellular function is to be maintained. Despite this, local reversible changes of extracellular and intracellular pH (pH_e and pH_i) are common occurrences, for example, during an increase in myocardial workload or a decrease in vascular perfusion, an extreme example being myocardial ischaemia. The talk will focus on three nodes within the Ca2+ signalling pathway of the ventricular myocyte, where pH is sensed and then transduced into a modified signalling code. (i) The dihydropyridine receptor (Cav1.2 protein) responsible for the L-type Ca2+ current (I_{Ca,L}) displays a steep differential sensitivity to pH_e where a fall is inhibitory, and to pH_i, where a fall is excitatory. These effects can be additive and are often overlaid by an additional secondary influence on the Cav1.2 channel caused by pH-induced changes of diastolic Ca2+.

(ii) Sarcolemmal Na+/H+ exchange (NHE) and Na+ -HCO3 cotransport (NBC) sense a fall of pH and export acid from the cell, while importing Na+ ions. The resulting rise of Na+ by acting on sarcolemmal/t-system Na+/Ca2+ exchange (NCX), raises Ca2+, ultimately in the lumen of the sarcoplasmic reticulum, but far less noticeably within bulk cytoplasm. The SR load results in an increased Ca2+ release during an action potential, thus increasing the Ca2+ transient (CaT), and hence contraction.

(iii) Diastolic cytoplasmic Ca2+ is, surprisingly insensitive to the elevation of Na+, induced by NHE/NBC during acidosis. There is an acid-induced rise of diastolic Ca2+, but this is independent of NHE/NBC activity. It is caused by Ca2+ release from local sources within the myocyte. The resulting elevation of Ca2+ maps fairly precisely onto any local, spatial variation of cytoplasmic pH. The mechanism that induces these pH-dependent Ca2+ microdomains is an active process, relying on the magnitude of the local H+ non-uniformity and upon ambient levels of ATP generated by mitochondrial and glycolytic metabolism. The possible mechanism involved will be explored in the lecture.

The integrated response of the myocyte to the pH-sensing and transduction system will be demonstrated by coding the system’s parameters into computational models of ventricular myocyte Ca signalling and the action potential. One insight is that intracellular acidosis induces intracellular Ca2+ loading via multiple pathways while extracellular acidosis reduces this. The H+ ion emerges as a major controller of intracellular Ca2+ dynamics in the myocardium.

This work is supported by the British Heart Foundation

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The chloride-bicarbonate exchanger AE1 is well characterized at one of its two major sites of expression, the red cell membrane (eAE1), where a number of important cytoskeletal interactions have been reported. In the kidney, requirements for its activity and regulation are somewhat different, since its major location is basolaterally in polarized epithelial cells (kAE1). Additionally, the N-terminus of kAE1 is significantly shorter than that of eAE1, so a number of cytoskeletal binding motifs are absent.

Mutations in AE1 are associated with an autosomal dominant human acidotic phenotype characterized by abnormal AE1 targeting. We have examined the C-terminal tail of AE1 (AE1C) for protein-protein interactions and identified several, includ-
Renal acid excretion – mechanisms and diseases
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The kidney fulfills a central role in maintaining and controlling systemic acid-base homeostasis. Three major mechanisms are operative: 1) reabsorption of filtered bicarbonate, mostly achieved by the proximal tubule, 2) ammoniagenesis in the proximal tubule regenerating bicarbonate used to buffer protons from metabolism and generating ammonium ions for renal acid excretion, 3) active secretion of protons and de novo generation of bicarbonate along the collecting duct. This talk will focus on the latter two processes highlighting mechanisms in the proximal tubule contributing to ammoniagenesis and transport pathways in the collecting duct that mediate ammonia and proton excretion.

Ammoniagenesis in the proximal tubule requires glutamine as substrate. Glutamine import into the cells of the proximal tubule is mostly mediated by the basolateral SNAT3 amino acid transporter, glutamine is then taken up by mitochondria and metabolized to yield ammonium and bicarbonate. The import of glutamine and its subsequent metabolism is highly regulated and stimulated by acidosis in a coordinated manner involving SNAT3 and the subsequent enzymes PDG, GDH, PEPC and components of the Krebs cycle and gluconeogenic pathway. A mouse model lacking SNAT3 shows reduced urinary ammonium excretion and mice die around 20 days after birth.

Final excretion of ammonia into urine is achieved by the cells lining the collecting duct. There, ammonia and protons are secreted in parallel. We have shown that the rhesus protein RhCG is expressed on basolateral and luminal membranes of these cells and that genetic ablation of RhCG in mice impairs urinary ammonium excretion. On the cellular level, microperfusion experiments of isolated collecting ducts demonstrated that RhCG mediates NH3 but not NH4+ transport and is the limiting step on the luminal membrane. On the basolateral side, RhCG contributes to NH3 uptake but other pathways for NH3 and NH4+ exist. NH3 in urine is trapped after titration to NH4+. Protons are excreted by the action of H+-ATPases, multisubunit pumps that are highly regulated by many factors including acid-base status and the renin-angiotensin-aldosterone system. Interestingly, one accessory protein of the pump serves also as receptor for (pro)renin. Mutations in the B1 and a4 H+-ATPase subunits cause distal renal tubular acidosis in humans. Similarly, mice lacking the B1 subunit have a reduced ability to acidify urine while the B2 isoform compensates partially for the loss of B1. However, not all functions of B1 are compensated and a trafficking defect of pumps lacking the B1 subunit is observed. Newly generated bicarbonate in the collecting duct is released by the AE1 anion exchanger mediating exchange of bicarbonate or extracellular chloride. Mutations in this exchanger have been detected in patients with autosomal dominant forms of distal renal tubular acidosis. The absence of this transporter apparently affects not only the transport capacity of intercalated cells but also their differentiation as evident from AE1 KO mice and kidney biopsies from patients. A further important aspect of renal acid excretion along the collecting duct is the adaptive remodelling of the cellular profile of the collecting duct in response to alterations of systemic acid-base balance. A higher systemic acid load causes an increase in the (relative) abundance of acid secretory type A intercalated cells whereas a higher alkali load reverses this process and enhances the (relative) number of type B bicarbonate secretory cells. Stimulation of latter cells may also occur with changes in chloride balance linking functionally acid-base and chloride (salt) homeostasis. The change in intercalated cell subtypes may be via interconversion of functional subtypes or may also involve regulated proliferation of differentiated intercalated cells as suggested by immunohistochemistry identifying differentiated type A or B intercalated cells with various markers of active DNA synthesis and cell proliferation. How this process is regulated remains elusive to date but may involve hormones and factors involved in mediating the stimulation of renal acid excretion during acid loads.

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are no in vivo data for the loop of Henle, but in vitro experiments have shown that stimulation of P2Y2 receptors and/or an unidentified P2X receptor inhibit Na⁺-K⁺-2Cl⁻ co-transport in the thick ascending limb, involving NO release. In the collecting duct, P2 receptor activation (apical and/or basolateral) has been shown to inhibit K⁺ channel activity, vasopressin-stimulated water reabsorption, and ENaC-mediated sodium reabsorption. While still not completely resolved, pharmacological profiling and experiments in transgenic mice suggest that P2Y2 is the receptor subtype responsible for these effects, although there seem to be important species differences in P2 receptor distribution and function - yet another feature of this system that has to be considered. Finally, P2-mediated actions may have a significant pathophysiological role, particularly in renal parenchymal and vascular injury (P2X7), and in renal cyst formation (P2Y2 and/or P2X7). However, there is still much to understand about the physiological/pathophysiological role(s) of this primitive and fascinating autocrine/paracrine system, and it is likely to provide a novel therapeutic target in the future.


I am grateful to Dr David Shirley for his help in preparing this abstract.

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The epithelial sodium channel (ENaC) is localized in the apical membrane of epithelial cells and is the rate limiting step for sodium absorption in a number of epithelial tissues including the aldosterone-sensitive distal nephron, respiratory epithelia, distal colon, sweat and salivary ducts. In the kidney ENaC is critically important for the maintenance of body sodium balance [1]. This is evidenced by gain-of-function mutations of ENaC which cause pseudohypoaldosteronism type I (PHA1), symptoms without overt renal disease. Furthermore, ENaC may be a modifier gene in patients with CF [2]. Increased ENaC activity may also contribute to the pathophysiology of cystic fibrosis (CF). This is supported by the observation that airway-specific over-expression of the β-subunit of ENaC in mice causes airway surface liquid depletion and CF-like lung disease. Loss-of-function mutations of ENaC cause pseudohypoaldosteronism type I (PHA1), a disease characterized by renal salt wasting.

ENaC is a member of the ENaC/degenerin family of ion channels which also includes the acid sensing ion channel ASIC1. The recently published crystal structure of chicken ASIC1 suggests that ENaC is a heterotrimer. Each subunit of ENaC contains two transmembrane domains, a large extracellular domain, and short intracellular amino and carboxyl termini. Three well-characterized ENaC subunits (αβγ) are thought to form the trimeric channel. In humans an additional δ-subunit of ENaC exists which can functionally replace the α-subunit in heterologous expression systems. So far little is known about the physiological function of the δ-subunit which is expressed in a wide range of tissues.

The tissue-specific regulation of ENaC by hormones and other factors is highly complex [1]. A unique feature of ENaC regulation is its proteolytic processing thought to be critical for channel activation under physiological and pathophysiological conditions [2]. However, at present the precise molecular mechanism of proteolytic channel activation remains unclear. The channel is in its mature and active form in its cleaved state, but there is evidence for the presence of both cleaved and non-cleaved ENaC in the plasma membrane. Membrane-bound and/or secreted proteases activate ENaC by cleaving specific sites in the extracellular domains of the α- and γ-subunits but not the β-subunit. Putative cleavage sites for furin, prostasin, plasmin, and elastase have been described. Cleavage at these sites probably results in the release of inhibitory peptides. This presumably activates the channel by changing its conformation. We showed that cleavage of the γ-subunit is particularly important for the proteolytic activation of near-silent channels [3] and that the δ-subunit is also proteolytically processed [4].

Most of our knowledge about ENaC activation by extracellular proteases stems from studies in model system like Xenopus laevis oocytes and cultured cells. However, functional evidence is emerging that ENaC activation by extracellular proteases can occur in native tissue. Indeed, we demonstrated that trypsin can activate ENaC in microdissected mouse distal nephron [5]. In nephrotic syndrome filtered plasminogen is converted to plasmin by tubular urokinase-type plasminogen activator. We have shown that plasmin activates ENaC [6]. Thus, ENaC activation by plasmin may contribute to sodium retention in nephrotic syndrome. Moreover, ENaC activation by locally generated proteases may aggravate symptoms of CF during acute respiratory infections. Furthermore, ENaC may be a modifier gene in patients with CF. Indeed, ENaC polymorphisms with a gain-of-function effect have been identified in patients with atypical CF [7]. At present it is unclear why some gain-of-function mutations of ENaC cause Liddle’s syndrome while others cause CF-like pulmonary symptoms without overt renal disease.

Organ specific differences in proteolytic ENaC processing may be responsible for the development of different disease phenotypes. However, the (patho-)physiologically relevant proteases for ENaC activation, their regulation and their specific cleavage sites remain to be determined.


This work was supported by the Deutsche Forschungsgemeinschaft (SFB423: Kidney Injury, Pathogenesis and Regenerative Mechanisms, Project A12) and by the Johannes and Frieda Marohn Stiftung.

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BLINaC: a novel epithelial Na⁺ channel in the liver
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Brain Liver Intestine Na Channel (BLINaC) is an ion channel of the DEG/ENaC gene family with unknown function (ref. 1). In rodents, the blinac mRNA is expressed mainly in brain, liver, and intestine and to a lesser extent in kidney and lung (ref. 1); in humans, it is mainly expressed in the small intestine (ref. 2).

To explore the expression of the BLINaC protein in these tissues, we generated a polyclonal antibody against mouse BLINaC, which recognizes an antigen of the correct size in Western blots. We currently use this antibody to determine the cellular expression pattern of BLINaC, in particular in the GI tract.

Functional expression of rat BLINaC (rBLINaC) in Xenopus oocytes leads to small unselective currents that are only weakly sensitive to amiloride (ref. 1). rBLINaC is potently inhibited by micromolar concentrations of extracellular Ca²⁺ (Kᵢ = 10 ± 1.5 μM) and removal of Ca²⁺ leads to robust currents and increases Na⁺ selectivity of the ion pore (ref. 3). Strikingly, the species ortholog from mouse (mBLINaC) has an almost 250-fold lower constitutively active at physiological Ca²⁺ concentrations (ref. 1). In addition, mBLINaC is more selective for Na⁺ and has a 700-fold higher amiloride affinity than rBLINaC (Kᵢ = 7.1 ± 0.9 μM compared with 6.4 ± 1.7 mM) (ref. 3). A single amino acid in the extracellular domain determines these profound species differences (ref. 3).

These results suggest that rBLINaC is opened by an unknown ligand whereas mBLINaC is a constitutively open epithelial Na⁺ channel. This is consistent with the species difference and is still enigmatic.

Recently, we identified the fenamate flufenamic acid (FFA) and related compounds as agonists of rBLINaC. Application of millimolar concentrations of FFA to rBLINaC expressing oocytes induces a robust, Na⁺-selective current, which is partially blocked by amiloride.

We also discovered that rBLINaC and mBLINaC, similar to the related acid sensing ion channels (ASICs) but in contrast to the epithelial Na⁺ channel ENaC, are inhibited by micromolar concentrations of diarylamidines and nafamostat. Thus, we identified pharmacological tools that will help to characterize the function of BLINaC in native tissues. Sakai H et al. (1999) J Physiol 519, 323–333


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Proton-assisted amino acid transporters: novel intracellular regulators of mTORC1 signalling in health and disease
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Both endocrine insulin/insulin-like growth factors and local amino acids are known to regulate this cellular pathway. However, there are still significant gaps in our understanding of the PATs’ role in the regulation of TORC1, despite its central importance in disease.

We have investigated how local amino acids are sensed and can modulate TORC1 signalling by screening a wide spectrum of amino acid transporters under physiologically relevant conditions, using the fly as an in vivo model (Goberdhan et al., 2005). This study highlighted the Proton-assisted Amino acid Transporter (PAT/SLC36) family as having a particularly potent effect on TORC1-mediated signalling and cellular growth.

Furthermore, our work suggested that PATs may act as so-called transceptors; that is, molecules that look like transporters, but which actually signal independently of transport to downstream targets upon binding to substrate. There are several known examples of transporters in yeast, but only the PATs and the related SNAT amino acid transporters (Hyde et al., 2007) have been proposed to function via this mechanism in higher eukaryotic organisms to date.

We have now shown using both siRNA knockdown and over-expression approaches that the growth-promoting properties of the PATs are conserved in human cells (Heublein et al., 2010).

siRNA knockdown in MCF-7 and HEK-293 cells results in typically >60% reduction in RNA levels and >60% decrease in cell proliferation, as well as >50% reduction in levels of activated downstream targets of TORC1 signalling. Intriguingly, these studies also highlighted an intracellular pool of PATs as being responsible for TORC1-mediated growth stimulation. Indeed PATs are located on late endosomes and lysosomes (LELs), the same location to which mTOR is recruited on amino acid stimulation and which requires the Regulator-Rag GTPase complex (Sancak et al., 2008; Sanack et al., 2010).

More detailed biochemical analysis suggests that PATs are part of a protein complex, including the Rag GTPases, that we have called the ‘nuitre-some’, which acts as an intracellular amino acid sensor that interacts with TORC1 (see Figure 1). PATs would be predicted to export amino acids from LELs, but may also be able to detect amino acids on their cytosolic face, through which substrates would exit. This mechanism has been suggested for other amino acid transporters and transceptors (reviewed in Goberdhan, 2010), and it may help to explain how cytosolic amino acids like leucine, which are thought to be key regulators of TORC1, could interact with the sensing mechanism.

More recently, we have also started to assess the role of PATs in the cell’s response to starvation, and recovery from amino acid depletion. Furthermore, our in vivo data highlight the differential localisation of PATs at the cell surface and LELs in cells with different growth rates, and suggests that the subcellular distribution of PATs might regulate TORC1-dependent growth.

We conclude that PATs regulate a critical intracellular mechanism involved in amino acid sensing. Although protein structures of PATs suggest that they are transporters, it is unclear if transport is critical to key effects of the PATs on TORC1-mediated growth. Our data suggest that the regulation of PAT localisation is an important factor in the ability of this molecule to impact on TORC1-mediated growth and accumulation of intracellular PATs may help to isolate cells from detrimental changes in the extracellular nutrient environment. We propose that a
PAT-dependent mechanism may be particularly important for the growth and proliferation of cancer cells, which are more able to survive and grow under such conditions than normal cells, which may be more dependent on cell surface transporters for growth regulation.

Figure 1. Schematic diagram illustrating the proposed role of PAT-containing nutrientosomes in amino acid regulated TORC1 activation on late endosomes and lysosomes. Members of the Proton-assisted Amino acid Transporter (PAT/SLC36) family and Ragulator-Rag GTPase complex located on late endosomes and lysosomes (LELs) are involved in the amino acid (AA)-stimulated activation of target of rapamycin complex 1 (TORC1). In addition to endocrine regulation via the insulin/insulin-like growth factor (IGF) signalling cascade, intracellular amino acids, in particular leucine (leu), have also been implicated in modulating TORC1 activity. The PATs, which can directly interact with amino acids, may form part of an amino acid sensing complex or ‘nutrisome’ on the surface of LELs.

Goberdhan, DC, Meredith, D, Boyd, CAR and Wilson, C (2005) PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids Development 132, 2365-75.


Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids.

Cell 141, 290-303.


This work was supported by grants from Cancer Research Technology (C19591/A9093) and Cancer Research UK(C19591/A6181 and C7713/A6174).

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Surely there must be an “alternative”?

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With reduced budgets, space and staff numbers, many Physiology Departments and Faculties of Life Sciences are finding it increasingly more difficult to provide individual laboratory-based research projects for all of their final year students. Furthermore, given that less than 20% of these graduates go into research-based careers, is an individual research project still the “Gold Standard” or should we be offering alternative, non-laboratory based projects more suited to the majority of our graduates final career paths? The QAA Biosciences benchmark statement (QAA 2007) states that final year projects do not necessarily have to be laboratory based, they can be in areas not strictly related to research, e.g., in education or in the public understanding of science.

This presentation will discuss the range of alternative projects currently being offered both within the Faculty of Biological Sciences at Leeds and at other Institutions including enterprise, educational development, science and society, commercial, and survey projects. Using examples of Science and Society projects (where students create and deliver curriculum-enhancing teaching sessions in local schools) and survey projects (e.g., healthy lifestyle surveys of taxi drivers or middle-aged Rugby League fans), student achievements, the transferable skills developed through these projects and their academic equivalence to laboratory-based research projects will be demonstrated. The practicalities of providing alternative projects will also be discussed as will the benefits, both to the Department and to students. The latter include encouraging students to be enterprising and innovative, and to develop and utilise a different range of transferable skills to those required for laboratory based projects. They therefore enhance student employability and their future career opportunities. As a consequence, they are extremely popular with students. In 2010-11, 26% of Biomedical Sciences students at Leeds opted for an alternative project as their first choice of project.

QAA Biosciences Benchmark Statement (2007)
http://www.qaa.ac.uk/academicinfrastructure/benchmark/statements/biosciences07.asp

The projects described were developed, in part, through the award of a University of Leeds Teaching Fellowship to DIL

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Final year undergraduate project students can produce blended learning resources which enhance the academic performance of undergraduate physiology students

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Blended learning is becoming an increasingly important aspect of Higher Education, particularly as student expectations rise and institutions seek innovative ways to embed technology in teaching and learning (Sharpe et al., 2006). Final year undergraduate project students are a valuable resource for pro-
Mechanisms of Gating and Modulation in Voltage-dependent Sodium Channels

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Voltage-gated sodium channels are primarily responsible for generation of the upstroke of an action potential in nerves, muscles, and heart. They share a common molecular architecture with other members of the voltage-gated ion channel family and comprise a central ion-conducting pore surrounded by four voltage-sensing domains1-3. In response to a depolarization, the voltage-sensing domains of the sodium channels undergo conformational changes that are transduced to the pore domain. These conformational changes in the voltage-sensor result in the opening of central pore and this process is referred to as “electromechanical coupling”. The molecular and structural mechanisms involved in electromechanical coupling remain poorly understood.

Previous studies on voltage-gated potassium (KV) channels have suggested that the intracellular gating interfaces involving S4, S5 and S6 helices are crucial determinants of the cross-talk between the voltage-sensors and pores. We have been systematically investigating the role of these interfaces in the domain III of a Na+ channel by mutating them to tryptophan. Tryptophan substitution in this region is expected to perturb the tight packing interactions and thereby alter coupling between the pore and voltage-sensor. To assess the effects of site specific mutations on activation dynamics of the voltage-sensor and pore, we combined voltage-clamp fluorimetry and conductance measurements. The activation of the domain III voltage-sensors was measured by monitoring the voltage-dependent change in fluorescence of a dye attached to a substituted cysteine residue at the extracellular end of the S4 segment. The pore dynamics, on the other hand, was obtained by measurement of ionic currents. To further probe the role of these interfacial residues, we have examined the effect of these mutations on voltage-sensor modulation by local anesthetics.

We found that almost of half of these mutations cause an effect on voltage-sensor and pore dynamics. Most of these have similar effects on voltage-sensor movement and pore opening. In other words, they concurrently stabilized (or destabilized) the activation of voltage-sensor and pore opening. But a small number of high-impact mutants (7 out of 55) had opposing effects on voltage-sensor and pore. They stabilized the activation of the voltage-sensor while destabilizing pore opening (1). To understand the role of these residues, we considered canonical models of cooperativity and find that these residues are highly likely to be involved in both the resting state and activated state coupling interactions. These experiments were complemented by examining the effect of drugs that allosterically modify the voltage-sensor movement by binding to the pore. In the wild type sodium channel, binding of lidocaine causes a large hyperpolarizing shift in the activation of domain III voltage-sensor. We find that in all seven of the previously identified high-impact mutants, this tight correlation between drug block and voltage-sensor modification is disrupted (2). Together, these experiments allow us to identify the critical residues involved in electromechanical coupling at one of the intracellular gating interface of a sodium channel. Identification of residues involved in coupling is the first step towards understanding the physical mechanisms that govern electromechanical coupling in a voltage-dependent ion channel. Upon mapping the critical residues on a sodium channel structure obtained by homology modeling, we find that many of these residues occur either in or near the flexible hinges connecting the various helices. We speculate that these regions presumably behave as elastic hinges which mediate conformational coupling between voltage-sensor and pore in a voltage-dependent ion channel.
SA43

Voltage sensor movements in cardiac IKs channels suggest a mechanism for how KCNE1 affects KCNQ1 channels

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The delayed rectifier IKs potassium channel, formed by co-assembly of alpha (KCNQ1) and beta (KCNE1) subunits, is essential for cardiac function. Though KCNE1 is necessary to reproduce the functional properties of the native IKs channel, the mechanism(s) through which KCNE1 modulates KCNQ1 is unknown. Here we report measurements of voltage sensor movements in KCNQ1 and KCNQ1/KCNE1 channels expressed in Xenopus oocytes using voltage clamp fluorometry. KCNQ1 channels exhibit indistinguishable voltage dependence of fluorescence and current signals, suggesting a one-to-one relationship between voltage sensor movement and channel opening. KCN1 co-expression dramatically separates the voltage dependence of KCNQ1/KCNE1 current and fluorescence, suggesting an imposed requirement for movements of multiple voltage sensors prior to KCNQ1/KCNE1 channel opening. This work provides the first insight into the mechanism by which KCNE1 modulates the IKs channel and presents a novel mechanism for beta subunit regulation of ion channel proteins.

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SA44

Development of unnatural fluorescent amino acids to probe ion channel conformations

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Voltage-clamp fluorometry is a powerful tool to examine conformational changes in ligand- and voltage-gated ion channels. In particular, the technique has provided a wealth of information on electrophysiologically silent conformational changes. However, the many studies in the field using this technique were essentially limited to solvent-accessible domains of the ion channels, as it uses the covalent modification of an introduced cysteine side chain with an environmentally sensitive fluorophore. This approach has left large regions of membrane proteins off limits. We therefore aim to synthesize novel fluorescent amino acids that are exquisitely sensitive to their local dielectric environment and can be incorporated at virtually any position within the ion channel. This would be a tremendous step forward in the field, as it would enable us not only to address the question of conformational changes within the protein but also would open up new avenues to study ion channel physiology and trafficking. Unnatural amino acid side chains with fluorescent properties have been introduced into ion channels before, but progress was hampered by significant shortcomings: the amino acid was either not sensitive to its environment or expression levels were at least an order of magnitude too low to observe macroscopic fluorescent changes in vivo. Here we use our optimized in vivo nonsense suppression method to generate ion channels carrying (non-fluorescent) amino acid side chains at high enough expression levels to observe macroscopic fluorescent changes when using conventional cysteine-linked dyes. This is an important milestone as it confirms that the technique is capable of generating high enough surface expression to observe macroscopic fluorescent changes that report on conformational changes in ion channels. Furthermore, we provide experimental evidence for the successful incorporation of fluorescent amino acids such as the nitroaromatic amino acid N-methyl-aminonitrobenzenzo-1,3-diazole (NBD), which is sensitive to the local dielectric environment and can be expressed at high levels using our optimized in vivo nonsense suppression method. These results provide a significant forward towards observing macroscopic fluorescent changes from an unnatural fluorescent amino acid incorporated in an ion channel expressed in vivo.

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Fast voltage sensor movement and slow pore opening of hERG channels brought to light using Voltage-Clamp Fluorimetry

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Among ion channels involved in the cardiac action potential, hERG channels encode the alpha-subunit that underlies the rapidly activating delayed rectifier K+ current (IKr) in the heart. These proteins play a central role in cardiac action potential repolarization and therefore in the termination of cardiac systole. Their correct function is crucial for normal overall action potential duration and a standard QT interval of the surface electrocardiogram, as alterations in their functional properties or membrane incorporation (by genetic mutations or by secondary drug effects) play major roles in the development of long QT syndrome, a prolongation of the QT interval of the ECG associated with ventricular arrhythmias. The importance of hERG channels resides in their unusual gating, that are slow activation and deactivation, and much faster inactivation/recovery from inactivation. One debated and undetermined issue is whether S4 movement in hERG channels is slow, if of coupling of the gating mechanism to pore opening is.

Voltage-clamp fluorimetry has been successfully used to assess S4 movement in other Kv channels. The same technique was thus applied by attaching TMRM to different extracellular areas of hERG channels (S1-S2, S3-S4 and S5P extracellular linkers) and measuring voltage-dependent movements of hERG channels to elucidate slow activation/deactivation. Results show different profiles of fluorescence depending on the location where the dye is attached, but consistently relate to pore opening/G-V in terms of time course and voltage-dependence, whatever the linker studied, leading to the conclusion that a concerted channel rearrangement is responsible for or resultant of slow opening in hERG channels. Such a concerted movement of several parts of the channels might be due to critical interactions that occur during hERG gating, as it is suggested that interactions between: 1) extracellular linkers, 2) S4-S5 linker and S6 segment (2) and/or 3) between N-terminal and C-terminal regions (3), increase the amount of energy necessary to open/close hERG channels. Interestingly, one residue at top of S4 (E519C) showed a combined fluorescence signal. Adding functional mutations or membrane incorporation (by genetic mutations or by secondary drug effects) play major roles in the development of long QT syndrome, a prolongation of the QT interval of the ECG associated with ventricular arrhythmias. The importance of hERG channels resides in their unusual gating, that are slow activation and deactivation, and much faster inactivation/recovery from inactivation. One debated and undetermined issue is whether S4 movement in hERG channels is slow, if of coupling of the gating mechanism to pore opening is.

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Accessing the intracellular compartment to examine the conformational dynamics of the Na,K-ATPase

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The Na,K-ATPase is a critical protein for cellular homeostasis. It transport three sodium ions for two potassium ions across the plasma membrane and utilizes the energy from ATP hydrolysis to transport these cations against the chemoelectric gradient. By combining electrophysiology and fluorescence-based methods, we have previously been able to measure the conformational dynamics of the holoenzyme and to determine distance constraints between subunits of the enzyme (1, 2). In our current experiments, voltage clamp fluorimetry was used to monitor conformational changes associated with electrogenic partial reactions of the Na,K-ATPase after changes in the concentration of internal sodium or external potassium (3). To probe the effects of the internal sodium concentration on the sodium branch of the Na,K-ATPase, Xenopus laevis oocytes were depleted of sodium and then loaded with sodium using the amiloride-sensitive epithelial sodium channel. The potassium branch of the Na,K-ATPase was studied by exposing the oocytes to different potassium concentrations in the presence and absence of internal sodium to obtain information on the apparent affinity for external potassium. Our results provide evidence on the relationship between lowering the internal concentration of sodium and increasing the amount of external potassium. These experiments demonstrate that it is possible to examine how external and internal ligands affect the conformational equilibrium of the ion pump on the surface of whole cells.


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Dick, Jane and sympathetic activity: sex differences in autonomic control of blood pressure

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Sex differences in the risk of hypertension are well recognized: prior to the menopause, women have a decreased risk of hypertension and other cardiovascular diseases compared to men. Conversely, hypertensive disorders such as orthostatic hypotension are more common in young women. Even among normotensive individuals, women tend to have lower blood pressure, and lower peripheral sympathetic vasoconstrictor nerve activity (SNA) compared to men. These differences disappear (or are reversed) at menopause. Although protective vasodilator effects of estrogen on vascular function have been demonstrated, integrative mechanisms for sex differences in blood pressure control remain poorly understood.

In recent years it has become increasingly clear that inter-individual differences in mechanisms controlling blood pressure (sympathetic nerve activity, cardiac output, vascular responsiveness) provide important insight into its regulation. In this context, resting levels of SNA are extremely variable, and can show a range of as much as 7 – 10 fold in a healthy young population. It is an important (although initially puzzling) characteristic of SNA that in general there is no relationship between SNA and arterial pressure among young normotensive humans: people with higher resting SNA do not necessarily have higher blood pressure. This relationship becomes positive in older individuals, and is particularly strong in post-menopausal women; these latter factors likely contribute to the increased risk of hypertension with aging. The mechanisms for the lack of SNA-BP relationship in young people, and changes in these mechanisms as functions of sex and aging have been the focus of our recent investigations.

Among normotensive young men, a direct relationship exists between muscle SNA and total peripheral resistance (TPR), suggesting that muscle SNA is a good index of whole body vasoconstriction tone in this group. An inverse relationship exists between SNA and cardiac output, and men with higher SNA also show blunted vasoconstriction to adrenergic agonists. These latter observations have provided insight into the variable role of SNA in blood pressure control among young healthy men: men with higher SNA have lower cardiac output and less adrenergic responsiveness, thus “balancing” the potential pressor effects of high SNA.

Surprisingly, the SNA-TPR and SNA-cardiac output relationships do not exist in young women, suggesting that sympathetically mediated vasoconstriction is modified by other factors associated with female sex and/or sex hormones. Our recent data suggest that beta-adrenergic-mediated vasodilation may offset alpha-adrenergic vasoconstriction in young women, thus contributing to the differences in blood pressure regulation between sexes. In young women, systemic beta-adrenergic blockade augmented forearm vasoconstrictor responses to norepinephrine. Furthermore, the relationship between SNA and TPR (which was absent before beta blockade) became significant and positive with beta blockade. Importantly, these effects of beta adrenergic blockade were not observed in young men or in post-menopausal women, in whom significant, positive SNA-TPR relationships existed both pre- and post-beta blockade. Taken together, these data point to integrated autonomic mechanisms which differ between men and women in control of vascular resistance and blood pressure. The changes seen at menopause suggest that female sex steroids are important modulators of sympathetic neurovascular interactions in women, and that beta-adrenergic vasodilation is an important contributor to this modulation.

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underlies the sympathetic activation associated with these conditions however remains to be elucidated.

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SA149

Sex and Sympathetic Neural Firing Patterns in Humans

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The sympathetic nervous system contributes importantly to the regulation of beat-to-beat arterial blood pressure (ABP) via baroreflex control in humans. Bursts of muscle sympathetic nerve activity (MSNA) occur more frequently during spontaneous reductions of ABP and are typically suppressed during increases in ABP. These transient variations of MSNA appear to correspond most closely with diastolic arterial pressure (DAP) fluctuations. Recent studies demonstrate that the regulation of resting MSNA is sex-dependent, yet relations between MSNA firing patterns and DAP in men and women are not well defined. The purpose of the present work is to determine if DAP-MSNA relations, as well as firing patterns of integrated MSNA, differ in young, otherwise healthy men and women. Our findings reveal that men demonstrate higher DAP-MSNA coherence and an increased percentage of consecutive bursts (i.e., two or more bursts in successive R-waves) compared to women. Moreover, DAP-MSNA coherence is significantly correlated to percentage of consecutive bursts in men, but not women. Because DAP-MSNA relations at rest may not necessarily represent DAP-MSNA relations during orthostasis, we also examined DAP-MSNA coherence and percentage of consecutive bursts during orthostatic stress induced via progressive lower body negative pressure (LBNP). DAP-MSNA coherence is strong (i.e., r=0.5) and incrementally increases throughout progressive LBNP stages in men. In contrast, DAP-MSNA coherence is significantly lower in women during progressive LBNP, and does not demonstrate similar incremental increases as observed in men. Men demonstrate a higher percentage of consecutive bursts during baseline and all stages of LBNP, although the percentage of consecutive bursts increases similarly during progressive LBNP between sexes. In conclusion, men and women demonstrate different firing patterns of integrated MSNA that appear to be related to differences in DAP oscillatory patterns. Men tend to have more consecutive bursts which contribute to stronger correlations between DAP and MSNA at rest and during orthostatic stress. These findings may help explain why young women are more prone to orthostatic intolerance.

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SA50

Sex and Neural Cardiovascular Responses to Dynamic Exercise in Humans

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Previous studies have demonstrated that young men exhibit greater blood pressure responses to exercise compared to young women. The neural mechanism(s) involved in such augmented responses in men remain unclear; however, sex differences in arterial baroreflex regulation of blood pressure may be involved. Indeed, the arterial baroreflex is of fundamental importance for evoking and maintaining the normal cardiovascular adjustments to exercise via modulating autonomic nervous system activity to the heart and vasculature. In particular, a properly functioning arterial baroreflex is critical to regulate blood pressure during exercise in which it protects against excessive sympatho-excitation and hypertension. Presently, animal and human studies have reported inconsistent findings on arterial baroreflex control when comparing sexes. Furthermore, whether sex influences arterial baroreflex control of blood pressure during dynamic exercise has not been investigated. A unique aspect in women that requires consideration when comparing responses to men is the potential impact of fluctuations in ovarian hormones. Although estrogen has been suggested to contribute to sex-related differences, a systematic examination of the influence of estrogen and progesterone on arterial baroreflex function and exercise-induced pressor responses has not been performed in humans. As such, we will present preliminary data examining sex differences in carotid baroreflex control at rest and during exercise. Five second pulses of neck suction and neck pressure were applied to load (carotid hypertension) and unload (carotid hypotension) the carotid baroreflex, respectively at rest and during steady-state cycling at 50% heart rate reserve. In comparison to men, young women appear to be better able to defend against hypertensive challenges at rest, whereas during exercise women appear to more effectively buffer against hypotensive stimuli. Furthermore, increases in endogenous estrogen and progesterone appear to selectively augment carotid baroreflex-mediated blood pressure responses to hypotension without effect on carotid baroreflex responses to hypertension. Interestingly, carotid baroreflex control of heart rate was not affected by changes in estrogen and progesterone throughout the menstrual cycle either at rest or during exercise. Overall, these preliminary findings suggest clear sex differences in arterial baroreflex control of blood pressure both at rest and during exercise.

Supported by National Institutes of Health R01HL093167.

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Pregnancy and Sympathetic Neural Activity in Humans
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Hypertensive disorders of pregnancy affect up to 10% of all pregnant women in the United States, and the most severe form, preeclampsia, is the leading cause of maternal and fetal death or morbidity. Although the mechanisms for such complications are unclear, it has been proposed that either gestational hypertension or preeclampsia is associated with a hyperadrenergic state, which may contribute to the pathophysiology of these conditions. Sympathetic adrenergic control plays an important role in blood pressure maintenance in humans. Vaso-motor sympathetic activity was found to increase in normal pregnancy and to be even greater in women with gestational hypertension or preeclampsia in late pregnancy. However, it is unknown whether sympathetic hyperactivity develops early in normal pregnancy, remaining high throughout the entire gestation, or whether this sympathetic activation only occurs at term, providing the substrate for gestational hypertension or preeclampsia. We found in 11 healthy Caucasian women (24-35 years old) that during early pregnancy (within 8 weeks of gestation), supine resting muscle sympathetic nerve activity (MSNA) was markedly greater (i.e., approximately twofold) compared with that of pre-pregnancy, while paradoxically their blood pressure and peripheral vascular resistance appeared to be lower. This finding is counter to the prevailing wisdom regarding the neurohormonal adaptation to normal pregnancy, which suggests that sympathetic activation occurs only in late pregnancy, and to our knowledge, there are no published nerve recordings in early human pregnancy. Supine MSNA remained elevated, while blood pressure increased slightly during late pregnancy in these women. Within 10 weeks after delivery, supine MSNA returned to the level of pre-pregnancy. One woman developed gestational hypertension at term; during early pregnancy she had greater supine blood pressure, heart rate, and MSNA when compared to other early pregnant women with normal pregnancies. These preliminary results suggest that sympathetic activation may be a universal characteristic of normal pregnancy in humans, while women with gestational hypertension and preeclampsia may have a further increase in sympathetic neural activity during early pregnancy.

Supported by the NIH R21 grant (HL088184).

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Hypothalamic pathways regulating energy homeostasis
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The hypothalamus is one of the key areas in the regulation of energy homeostasis as it receives constant updates on metabolic state from the periphery, it integrates this information and ultimately initiates necessary adjustments. Although tremendous progress in understanding the hypothalamic pathways regulating metabolic balance has been made in recent years, much of the information on how hypothalamic neurons sense nutrient state and translate that information into adjustment of neuronal function and ultimately e.g. food intake remains poorly understood.

Here, we will briefly review some of the current state-of-the-art in our knowledge of the hypothalamic pathways regulating energy homeostasis, but then focus on our use of hypothalamic nutrient-sensing as a model in which to study the interplay between a neuron’s dynamically modified transcriptional state and its physiological function. We have identified an important role for the CREB co-activator CRTC2 (CREB-regulated transcription co-activator) in linking hypothalamic glucose-sensing to appropriate CREB-target gene expression (1). Using hypothalamic area-specific genetically modified mice we demonstrate that hypothalamic CRTC2 indeed links glucose-sensing with an appropriate transcriptional state which ultimately regulates food intake.


Research Councils UK, Lister Institute for Preventive Medicine, British Heart Foundation

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How peripheral satiety signals access and influence the brain
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Central nervous system (CNS) structures involved in the regulation of energy balance gather information from the variety of different peripherally derived signaling molecules that we now believe provide an integrated perspective of energy status of the organism. However, the existence of the blood brain barrier means that the CNS is theoretically unable to directly monitor many of these circulating signals such as adiponectin, amylin, cholecystokinin (CCK), glucose, ghrelin, leptin, and peptide YY (PYY) which do not freely diffuse across this barrier. A number of mechanisms have been suggested to play important roles in facilitating the ability of the CNS to monitor this essential sensory information. My presentation will describe briefly potential roles of vagal afferent signaling and peptide transporters in providing access routes for such information transfer, but will focus primarily on the potential roles of specialised CNS structures which lack the blood brain barrier known as the sensory circumventricular organs (CVOs). In particular I will highlight the complex sensory abilities of single CVO neurons in sensing multiple satiety signals and also describe the efferent projections of these neurons to essential autonomic control centres behind the blood brain barrier.

Supported by grants from the CIHR (Can), NIH (USA), and HSFO (Can).

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Five years ago, the protein Nucleobindin2 (NUCB2; Barnikol-Watanabe et al., 1994) was first suggested as a regulator of energy metabolism. Oh-I et al (2006) proposed that NUCB2 is cleaved into three protein fragments (nesfatin-1, -2 and -3) and showed that administration of nesfatin-1 into the brain results in anorexia and relative body weight loss. Mapping of NUCB2 in the brain revealed expression in several key metabolic control regions of the brain, including nuclei controlling feeding behaviour, but also endocrine and autonomic output, suggesting a role also in energy expenditure (Foo et al., 2008). Anatomical and biochemical data, however, are difficult to reconcile with processing of NUCB2 into fragments and secretion: NUCB2 immunoreactivity is concentrated in several confine cell bodies and absent from terminals, whereas western blot analysis to date has not revealed the existence of endogenous fragments corresponding to nesfatin-1.

There is considerable overlap between the signal molecule repertoire between the metabolic regulatory regions of the CNS and peripheral organs involved in energy balance. Recent work from several groups, including ours (Gonzalez et al., 2009; Stengel et al., 2009; Foo et al., 2010), has shown that this is true for NUCB2, which shows particular concentration in the insulin-producing beta cells of the human and rodent pancreas (but is absent from other islet cells). While fasting did not affect NUCB2 islet content, it was decreased by 50% in islets from Goto-Kakizaki (GK) rats, a model of type 2 diabetes. Curiously, levels in the GJ islets normalized with fasting. Release of NUCB2 from islets following glucose challenge (+23%) were, however, modest compared to the increase in insulin secretion (+716%), arguing against a major secretory role for NUCB2 in islets. These and other data suggest that NUCB2 plays a role in the regulation of energy metabolism across several glucoregulatory organs, but that this role may be exerted intracellularly, rather than as a cleaved and secreted messenger.


secretion from the pituitary gland and, by unique CNS mechanisms, activate the sympathetic nervous system. The relationship of those central actions to their effects on pancreatic islet cell function, in particular beta cell response to glucose challenge, will be described and those in vitro actions supplemented by a discussion of their effects on glucose tolerance in vivo. It will be suggested that both peptides provide the opportunity for not only the understanding of the physiologic mechanisms controlling energy balance but also the potential development of novel therapeutic approaches to the treatment of obesity and its attendant co-morbidities.


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The author’s work is funded by grants from the National Institutes of Health (HL-66023) and the American Heart Association (AHA 4470043).

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SA57

Vascular extracellular matrix proteins controlling endothelial and smooth muscle function

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The dystrophin-glycoprotein complex (DGC) is a multimember complex that connects the cytoskeleton of a muscle fiber to its surrounding laminin containing extracellular matrix. The main components of the DGC are dystrophin, dystroglycan and the sarcoglycan complex. Mutations in any of these components lead to various forms of muscular dystrophy and often cardiomyopathy. The DGC is not only expressed in skeletal and heart muscle cells but also in vascular smooth muscle cells as well as in endothelial cells. The DGC has been extensively studied in skeletal muscle, but its role in vasculature is less clear. I will discuss a few studies in which the roles of DGC components in vascular smooth muscle have been examined and how absence of vascular DGC impacts muscular dystrophy. For example, it has been demonstrated that sarcoglycan deficient animals display vascular dysfunction that contributes to development of muscular dystrophy and cardiomyopathy. However, it remains controversial how these vascular spasms arise. Moreover, dystrophin deficient smooth muscle contributes to the dystrophic phenotypes of Duchenne muscular dystrophy (the most common type of muscular dystrophy). Finally, we have recently in collaboration with Anna Hultgårdh-Nilsson begun to analyze the roles of laminins and DGC components in the atherosclerotic process. We have demonstrated that dystrophin deficiency in mice stimulates neointima formation. Hence, dystrophin in vascular smooth muscle cells may protect the vessel wall against injury and atherosclerosis. Conversely, boys with Duchenne muscular dystrophy may be more susceptible to the development of atherosclerotic lesions and may more easily develop restenotic lesions in response to angioplasty.

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SA58

Insights from proteomic analyses of adhesion signalling

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Multicellular life requires the formation of extracellular matrices and the sensing of the cell environment through regulated adhesive interactions. By coupling the binding of extracellular adhesion proteins to the assembly of intracellular cytoskeletal and signalling complexes, integrin receptors mediate the bidirectional transmission of mechanical force and biochemical signals across the plasma membrane. Integrin-based adhesion is highly dynamic, as cells must rapidly respond to changes in their environment by altering their migratory properties, gene expression profile and proliferation state. A detailed, integrative view of the dynamics of adhesion complexes would provide insight into the molecular mechanisms that control cell morphology, movement, survival and differentiation, but, as with other membrane receptor-associated signalling complexes, integrin adhesion complexes have been refractory to isolation due to their instability and inaccessibility. A literature-curated model for the composition of adhesion complexes has revealed massive complexity (1), but despite decades of work, the global composition and mechanisms of regulation of integrin-associated protein complexes are relatively poorly understood. We reasoned that there was a need for technologies that enable systematic, proteomic analysis, and accordingly we have developed a methodology for the affinity isolation and mass spectrometric analysis of integrin adhesion complexes (2). In follow-up studies to this original publication, the isolation of stabilised complexes associated with multiple integrin receptor–ligand pairs, and detailed quantitative analyses of their composition at multiple time points and in different receptor activation states, have been carried out. Our analyses have defined temporal profiles of integrin-associated protein complexes during the initial stages of cell adhesion, and compared the complexes that are assembled by integrins occupied either by ligands or by monoclonal antibodies that freeze receptor conformation in different stages of activation. Hierarchical clustering and protein interaction network analyses reveals distinct dynamics of protein modules relevant to cell adhesion processes. Although we should not underestimate the scale of the task, the development of this workflow now permits the molecular dynamics of adhesion complexes to be measured directly and presents an entry point for quantitative, systems-level analyses of adhesion signalling in health and disease.

studies of the elastin network demonstrate that it constrains arterioles longitudinally and when it is selectively compromised there is a resulting lengthening of the vessel. Collectively, knowledge of the role of the ECM in vascular control and the three-dimensional architecture of the extracellular matrix components within the vessel wall will help provide important new insights into our understanding of the structure-function relationships that exists in small arteries. Support to GAM NIH1PO1HL095486.

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SA59

Integrins and matricryptins in the control of microvascular tone and structural adaptation

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The extracellular matrix (ECM) is composed of a variety of proteins including collagen, elastin, laminin, fibronectin, vitronectin, glycoproteins, and proteoglycans. Structurally, these proteins provide a mechanically dynamic and adaptable scaffold that is involved in processes including cell growth, differentiation, migration and contraction and remodeling. In consideration of the vasculature, we have demonstrated that the ECM can also present vasoactive signals to cells of the vascular wall that through outside-in mechanisms that convey soluble or mechanical signals to both vascular smooth muscle and endothelial cells. As examples, we and others have shown ECM fragments containing the Arginine-Lysine-Aspartic acid (RGD) sequence are vasoactive and can reduce vascular tone producing arteriolar dilatation as well as inhibit the vascular myogenic response to step increases in intravascular pressure. This evidence has led to hypotheses that integrins may act as injury receptors for soluble RGD containing fragments of ECM proteins that might present during vascular or tissue injury. Additionally, the integrins are thought to act as receptors that can convey mechanical signals from the ECM to the cell. Mechanistically integrins interact with a number of signaling pathways including ion channels important for vascular function. Fibronectin binding through cell surface integrins modulates the open probability of smooth muscle cell ion channels (voltage-gated Ca2+ channels and large conductance Ca2+-activated K+ channels) and can induce local cellular contractions at the level of a single focal adhesion. Despite our knowledge of the ECM in vasomotor control and vascular cell signaling, relatively little is known of the complexities of the in situ arrangement between specific ECM proteins and arteriolar smooth muscle cells. Evidence now exists to demonstrate that VSMC are rapidly adaptable in the sense of being able to adjust their position within the vascular wall during periods of prolonged vasoconstriction. This has been viewed as a form of acute remodeling and appears to involve alterations of the VSMC cytoskeleton as well as cell-ECM relationships. Given these examples, it is becoming increasingly clear that understanding the structural arrangement of the vessel wall ECM, particularly at the microvascular level, is vital for determining how local mechanical forces are transmitted, sensed and responded to and how vessels are able to interact with the ECM to alter vessel diameter. Recent studies in our laboratories have used three-dimensional confocal/multiphoton microscopy as a means to resolve structural details related to ECM protein distribution within the arteriolar wall. Our studies of elastin have revealed an elaborate network of organized fibers that course through the vascular wall. Functional studies of the elastin network demonstrate that it constrains arterioles longitudinally and when it is selectively compromised there is a resulting lengthening of the vessel. Collectively, knowledge of the role of the ECM in vascular control and the three-dimensional architecture of the extracellular matrix components within the vessel wall will help provide important new insights into our understanding of the structure-function relationships that exists in small arteries. Support to GAM NIH1PO1HL095486.

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SA60

Control of shear stress induced endothelial growth factor release by extracellular matrix proteins

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We have shown previously that the shear stress induced augmentation of endothelial NO synthase expression is dependent on the composition of the extracellular matrix. This observation led us to suggest that the extracellular matrix may decisively modulate the responsiveness of endothelial cells to shear stress. The composition of the matrix can be modulated by an altered cellular synthesis of matrix proteins as well as by the effects of proteases released from endothelial cells or from the extracellular matrix. Indeed, work of several laboratories has demonstrated that endothelial cell exposed to shear stress may alter the release of proteases, thereby altering the extracellular matrix composition in a way that may lead to cell activation via integrins. As an example, we could demonstrate that exposure to shear stress (16 dyn/cm2) elicited the release of a protease with elastolytic properties from porcine aortic endothelial cells (PAEC). The protease dependent activation of the integrin αvβ3, and a signalling cascade involving p38 and Hsp 27, ultimately resulted in the release of fibroblast growth factor-2 (FGF-2) into the supernatant. Though the released protease is not identical with known elastases of identical molecular weight (including neutrophil elastase and proteinase 3) it was found that addition of a pancreatic elastase induced, similarly as shear stress, the release of FGF-2. We, therefore, studied whether treatment of the extracellular matrix of cultured PAECs with exogenous elastase could activate regulatory compounds of the αvβ3 related signalling pathway as shear stress. To this end, PAECs grown to confluence on a matrix which initially consisted of collagen I were stimulated for 60 minutes with pancreatic elastase (0.5 U/ml) and afterwards stained for laminin, elastin or fibronectin. Interestingly, while the macroscopic distribution of elastin (as studied by immunofluorescence) was not altered during elastase incubation, the extracellular network of laminin and especially that of fibronectin was clearly altered suggesting that these matrix alterations could possibly lead to re-clustering of integrins. Indeed, addition of exogenous elastase led to a transient disappearance of existing focal contacts followed by a distinct topological re-arrangement. This effect correlated with time dependent changes of Tyr phosphorylation of focal adhesion kinase (FAK). Inhibition of αvβ3 integrins by an antibody (abciximab) did prevent p38 phosphorylation and FGF-2 release both, under conditions of shear stress and exposure to the exogenous elastase. We, therefore, conclude that a significant component of shear stress induced signalling events occurs at the abluminal side of endothelial cells via matrix dependent integrin signalling. This offers a novel pathway regulating the
apparent responsiveness of endothelial cells to shear stress which may play a prominent role in growth factor dependent vascular remodelling processes elicited by shear stress.

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SA61

A proangiogenic role of the vascular basement membrane

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Endothelial integrins that bind to provisional extracellular matrix (ECM) have been extensively studied, while the role of integrins involved in adhesion to vascular basement-matrix (BM), such as laminins, is less clear. Laminin-binding integrins such as α6β1 and α3β1 are considered important exclusively for the process of endothelial tube stabilization and their role in regulating sprouting angiogenesis is uncertain. We demonstrate that α6 integrin is up-regulated by angiogenic factors in endothelial cells (EC) and is high expressed in the angiogenic vasculature. In EC, where α6 integrin is down-regulated, defects in the ability to migrate and form tubular structures on laminin-containing matrix are prominently displayed. However, when similar experiments are performed on ECM ligands other than laminin, the α6 integrin down-regulation did not modify the EC response.

Moreover, our results provide evidence that α6 integrin plays an unexpected role in adult pathological angiogenesis, showing that blockade of α6 integrin inhibits vessels formation both in CAM and in mammalian tumor transgenic model. The reduced tumor vessel diameter, together with the relative abundance of pericytes in mice treated with anti-α6 integrin antibody, suggest that the increase of α6 on EC contribute to the morphological and functional defects described in tumoral vessels.

Furthermore, we observe that α6 integrin localizes to basal surface of EC and specifically in podosomes. Podosomes are specialized plasma-membrane microdomains that combine adhesive and proteolytic activities to spatially restricted sites of matrix degradation. We demonstrate the presence of α6 but not α3 integrin in the ring structure that surrounds the podosome core, both in EC in vitro and in aortic vessels in vivo. Moreover, laminin reduces α6 integrin localization to podosomes in a concentration-dependent manner.

In conclusion, our results demonstrate that α6 is expressed on angiogenic EC in both culture and tumors, and it plays an important role in vascular sprouting and tumoral angiogenesis, regulating migration and proteolytic activity of EC.

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Physiological significance and activation of the epithelial calcium-dependent chloride channel TMEM16A

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Endogenous Ca2+ activated Cl- channels (CaCC) demonstrate biophysical and pharmacological properties also found in cells overexpressing TMEM16A (anoctamin 1, ANO 1), a protein that has been recently identified as CaCC. Proteins of the anoctamin family (TMEM16A-K, anoctamin 1-10) are expressed abundantly. The number of reports demonstrating their physiological and clinical relevance is quickly rising. TMEM16A null mice exhibit severe defects in epithelial transport along with tracheomalacia and death within one month after birth. TMEM16-proteins have also other interesting properties that may be related to cell swelling, control of cell volume, and apoptosis.

Available data suggest that TMEM16-proteins form stable dimers. However, despite its outstanding physiological significance, the mechanisms for activation of TMEM16A are unclear. TMEM16A is activated upon increase in intracellular Ca2+, but we don’t know whether Ca2+ binds directly to the channel or whether additional components are required. We demonstrate that TMEM16A is strictly membrane localized and requires cytoskeletal interactions to be fully activated. Despite the need for cytosolic ATP for full activation, phosphorylation by protein kinases is not required. In contrast, the Ca2+ binding protein calmodulin appears indispensable and interacts physically with TMEM16A. Openers of small and intermediate conductance Ca2+ activated potassium channels like 1-EBIO are known to interact with calmoduline and also activate TMEM16A. Our present results suggest the use of these compounds for activation of electrolyte secretion in diseases such as cystic fibrosis.

Work supported by DFG SF699-A7, Mukoviszidose e.V. (Projekt-Nr.: 502/10) and TargetScreen2 (EU-FP6-2005-LH-037365). Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

The role of anoctamins in olfaction

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In vertebrate olfactory transduction a calcium-dependent chloride efflux greatly amplifies the odorant response. Indeed, the binding of odorants to receptors in the cilia of olfactory sensory neurons activates a transduction cascade that involves the opening of cyclic nucleotide-gated channels and the entry of calcium in the cilia. Calcium activates a chloride current that, in the presence of a maintained elevated intracellular chloride concentration, produces an efflux of chloride ions and amplifies the depolarization. Despite the relevant physiological role played by calcium-activated chloride channels in olfactory transduction, as well as in several other physiological processes, their molecular identity is still unclear. We showed by immunohistochemistry that anoctamin2/TMEM16b is expressed in the ciliary layer of mouse olfactory sensory neurons and performed an extensive functional comparison using both inside-out and whole-cell voltage-clamp techniques to record currents in the ciliary region of mouse isolated olfactory sensory neurons and in HEK293 cells transfected with anoctamin2/TMEM16b. Our findings support the hypothesis that anoctamin2/TMEM16b is a promising candidate to be part of the native olfactory channel, which contributes to the chloride-based amplification in olfactory transduction.

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Tmem16a expressed by interstitial cells of Cajal contributes to pacemaker activity and neural responses in visceral smooth muscles

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Interstitial cells of Cajal (ICC) are unique cells that generate electrical pacemaker activity in gastrointestinal (GI) smooth muscles. Conductances, such as Ca2+-activated Cl-- channels (CaCC) and non-selective cation channels (NSCC) have been proposed to mediate pacemaker activity. We characterized the expression of CaCC in gastrointestinal (GI) muscles. Anoctamin 1 (ANO1), encoded by Tmem16a was originally found to be highly expressed in ICC in a microarray screen. Several splice variants of Tmem16a are expressed in GI muscles, as well as other paralogues of the Tmem16 family. ANO1 protein is abundant and specifically distributed in all classes of ICC in murine, non-human primate (Macaca fascicularis) and human GI tracts. The effects of CaCC blocking drugs, niflumic acid and 4,4′-disothiocyanato-2,2′-stilbene-disulfonic acid (DIDS) were tested on spontaneous slow wave activity in intact muscles of mouse, primate, human small intestine and stomach. Block of CaCC blocked slow waves in a concentration-dependent manner. The mechanism of pacemaker activity was investigated in cells isolated from transgenic mice with constitutive expression of a green fluorescent protein (copGFP) in ICC. Depolarization of ICC caused large amplitude inward currents that were due to a Cl--selective conductance as evaluated by reversal of tail current analysis. Removal of extracellular Ca2+, replacement of Ca2+ with Ba2+, or extracellular Ni2+ (30 μm) blocked the inward current. Single Ca2+-activated Cl-- channels with a unitary conductance of 7.8 pS were resolved in excised patches from ICC. Properties of the single channels were similar to ANO1 channels (8 pS) expressed in HEK293 cells. The inward current was blocked in a concentration-dependent manner by niflumic acid (IC50= 4.8 μm). Under current clamp conditions, transient depolarizations occurred spontaneously, and these events were blocked by niflumic acid. In intact muscles slow waves failed to develop in mice with Tmem16a deactivated (Tmem16atm1Bdh/tm1Bdh). This was not a delayed developmental error as pacemaker activity did not develop many days after birth. Absence of ANO1 and electrical pacemaker activity did not inhibit the development of ICC networks, because normal appearing cells were abundant. These data demonstrate that ICC have a prominent CaCC that contributes to the generation of pacemaker activity.
Mechanical Properties and Neural Control of Human Hand Motor Units
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Motor units serve both as the mechanical apparatus and the final stage of neural processing through which motor behaviors are enacted. Therefore, knowledge about the contractile properties and organization of the neural inputs to motor units supplying finger muscles is essential for understanding the control strategies underlying the diverse motor functions of the human hand. In this review, basic physiological properties of motor units residing in various human hand muscles are described. Information is considered as to whether or not motor units that reside in multitendoned finger muscles exert force on more than one tendon. In addition, the distribution of corticospinal inputs to motor nuclei supplying different hand muscles is described. Evidence for substantial divergence of such input across certain motor nuclei is presented and the functional significance of such neural coupling for the control of hand movements is discussed.

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SA67

The Range of Constraints for Control of the Human Hand
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The hand contains more than 20 muscles and joints which perform a multitude of jobs, from writing and gesture to the use of tools and playing musical instruments. The human hand is sometimes portrayed as the pinnacle of evolutionary development, yet both its biomechanics and neural control have major limitations. These occur at a peripheral level (muscle, joint and tendon) and centrally at spinal, cortical and other supraspinal sites. As examples, in the periphery, movement across the full range of possible joint space is limited by the force-length properties of the extrinsic muscles. Further, when the hand is positioned with all but one finger fully flexed, then that finger cannot be moved by volition at its distal joint – it is disengaged from central drive and effectively paralysed. At the level of the muscle-tendon unit, especially for the multitendoned extrinsic flexor and extensor muscles, there can be constraints introduced by the architecture of the motor unit territories, the connections between tendons, and there is the potential for some lateral transmission of force, both within and perhaps between muscles. At a central level, it has long been recognised from studies in non-human primates that there are limits in the ability of the corticospinal system to activate the motoneurones of individual muscles. The degree of common descending drive to muscles can be examined in humans by measurement of the correlated firing of pairs of motor units. This and other experimental approaches suggest that the capacity for selective activation of a muscle is greater for the distal intrinsic muscles compared to the proximal extrinsic muscles. In addition, there have been a number of studies of the degree to which forces can be produced voluntarily at joints of one finger with or without forces being produced involuntarily in the neighbouring digits. These have led to two concepts: ‘enslavement’ of force when force inadvertently appears at unintended digits (even in weak efforts); and ‘deficits’ of force when maximal voluntary force is less than expected when more than one digit is used. These properties of the hand motor system differ for movements into flexion and extension. They favour preferential extension of the fingers together to lift them from an object but favour flexion of individual fingers to contact an object. This organisation of neural drive and force distribution in humans allows the specialised tactile surface of the finger pads to explore and grasp objects. For common operations, the hand moves seamlessly through joint ‘space’ and we have little awareness of the neuromechanical limitations imposed at different levels. This highly evolved ability likely reflects the flexibility of corticofugal output to the motoneurone pools.

National Health and Medical Research Council

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SA66

Changing circuits that control the fingers: Dissociating motor cortex from the motor
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In understanding how the motor cortex controls the fingers, we tend to make the simplifying assumptions that the functional role of a given cortical neuron and its effect on a particular motoneuron pool both remain relatively constant. If so, then it should be possible to sum the activity of multiple cortical neurons, each weighted by the strength of its effect on the motoneuron pool, and predict the activity of their common target muscles. In actuality, however, such simple summing of cortical neuron activity accounts for muscle activity only partially. One possible explanation would be that the effect of a given cortical neuron on a particular motoneuron pool is, in fact, variable. We tested this hypothesis by rewarding monkeys for discharging the same cortical neuron in combination with different target muscles. During intense co-activation of a given cortical neuron with various muscles, the amplitude of spike-triggered average effects between the cortical neuron and any given muscle often varied substantially. In some cases, the pure post-spike effect of a cortico-motoneuronal (CM) cell was clearly present during some epochs, but absent during others. The throughput of CM connections thus appears to be variable. If a CM cell’s effect on muscles is variable, then can the function of cortical neurons vary as well? When neuron firing rates are used for closed-loop control of a brain-machine interface, many neurons change their preferred direction, limb movements diminish, and eventually muscle contractions stop. Cortical neurons then continue to discharge in dissociation from the body movements they previously appeared to control. We therefore are beginning to examine factors that determine whether motor cortex neurons can be combined into small ensembles, dissociated from finger move-
Spontaneous Ca$^{2+}$ release is normally in the form of a Ca$^{2+}$ from the sarcoplasmic reticulum (SR) during diastole. Arrhythmias. The cause of DADs is the spontaneous release of ventricular cardiomyocytes are thought to be the cause of arrhythmias. Late coupled or delayed after-depolarisations (DADs) in ventricular cardiomyocytes are thought to be the cause of arrhythmias. The cause of DADs is the spontaneous release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) during diastole. Spontaneous Ca$^{2+}$ release is normally in the form of a Ca$^{2+}$ wave that starts at a discrete part of the cardiomyocytes and propagates along the length of the cell. Understanding the subcellular mechanisms of these spontaneous events is critical in designing pharmacological approaches to block the arrhythmic event. Initially, this talk will describe work designed to quantify the Ca$^{2+}$ fluxes associated with the Ca$^{2+}$ wave based both on intracellular and intra-SR Ca$^{2+}$ measurements. The data shows how the spontaneous release process copes with moderate levels of cellular Ca$^{2+}$ load, in particular the role of Ca$^{2+}$/calmodulin-dependant protein kinase (CaM kinase) in the modulation of Ca$^{2+}$ waves. At very high cellular Ca$^{2+}$ values, sustained contraction and cell death is temporarily prevented by mitochondrial Ca$^{2+}$ uptake. The conventional view of the Ca$^{2+}$ wave is that it originates as a local Ca$^{2+}$ release from a cluster of ryanodine receptors (RyR) located within the dyadic cleft of the cardiomyocyte. The local release event (Ca$^{2+}$ spark) initiates release from an adjacent cluster via the process of Ca$^{2+}$-induced Ca$^{2+}$-release, and this “fire-diffuse-fire” process is thought to explain Ca$^{2+}$ wave propagation. In the final section of the talk, the issue of Ca$^{2+}$ wave propagation is explored in more detail, in particular the evidence for the role that RyRs out with the dyad may have in the propagation of Ca$^{2+}$ waves. The data supporting the role of extra-dyadic RyRs also suggests strategies to suppress Ca$^{2+}$ wave occurrence (and the associated arrhythmic event) without significantly affecting the Ca$^{2+}$ release from the main clusters of RyRs within the dyadic cleft. Inhibition of non-dyadic RyRs may be part of the explanation for the ability of the novel candidate antiarrhythmic drug K201 (formerly JTV519) to suppress spontaneous Ca$^{2+}$ release.

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SA69

What leads to recovery of hand function after stroke?  
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Stroke often leads to impairment of hand function. Over the following months a variable amount of recovery can be seen. The evidence from animal and human studies suggests that reorganization rather than repair is the key. Surviving neural networks are important for recovery of function and non-invasive techniques such as functional magnetic resonance imaging allow us to study them in humans. For example, initial attempts to move a paretic limb following stroke are associated with widespread activity within the distributed motor system in both cerebral hemispheres, more so in patients with greater impairment. Disruption of activity in premotor areas using transcranial magnetic stimulation prior to movement can impair motor performance in stroke patients but not in controls suggesting that these new patterns of brain activity can support recovered function. In other words, this reorganization is functionally relevant. More recently, research is directed at understanding how surviving brain regions influence one another during movement. This opens the way for functional brain imaging to become a clinically useful tool in rehabilitation. Understanding the dynamic process of systems level reorganization will allow greater understanding of the mechanisms of recovery and potentially improve our ability to deliver effective restorative therapy.

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SA71

Role of CaMKII in hypertrophy and heart failure  
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Heart failure (HF) is associated with systolic and diastolic dysfunction caused by abnormalities in intracellular Ca$^{2+}$-handling and structural remodeling. Several targets accompanied with the remodeling processes have been identified: The sarcoplasmatic reticulum (SR) Ca$^{2+}$-ATPase protein levels were found to be downregulated and paralleled by a reduced SR Ca$^{2+}$-uptake in the failing heart. In contrast, the sarcosomial Na$^+$/Ca$^{2+}$-exchanger (NCX) was found to be increased (protein level as well as activity) thereby even more effectively competing for Ca$^{2+}$ removal from the cytosol with the reduced SR Ca$^{2+}$-ATPase activity. This reciprocal expression of the two most important intracellular Ca$^{2+}$-transport proteins occurs during the pathogenesis of heart failure. The net effect is an impaired SR Ca$^{2+}$-loading which leads to smaller intracellular Ca$^{2+}$ transients and elevated diastolic Ca$^{2+}$ levels. The result of impaired contractility such as reduced contractile force and diastolic dysfunction are well accepted determinants in the failing heart.

Ca$^{2+}$-homeostasis of cardiac myocytes is regulated by phosphorylation of several key proteins thereby controlling Ca$^{2+}$-fluxes but may also cause ionic disruption in heart failure. A major regulatory kinase of Ca$^{2+}$-handling in cardiac myocytes is the Ca$^{2+}$/calcmodulin-dependent protein kinase II (CaMKII). It is a protein kinase that modulates several important Ca$^{2+}$-dependent regulatory proteins in myocytes, such as the SR
Calcium handling abnormalities in inherited arrhythmias

S.G. Priori

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease associated with cardiac arrest in the paediatric population. The disease was first described as a novel clinical entity by 1995. With the advancements of genetics the discovery of the molecular substrate of the showed that CPVT results from inherited abnormalities of intracellular Ca2+ regulation caused by dominant mutations in the RYR2 gene, encoding the cardiac Ca2+ release channel (ryanodine receptor isoform 2 [RyR2]) and by recessive mutations in the CASQ2 gene, encoding cardiac calsequestrin isoform. The discovery of the molecular substrate of CPVT has fuelled basic science studies to characterize RYR2 and CASQ2 mutations in vitro and in vivo, leading to important advancements in the understanding of intracellular Ca2+ regulation and its relevance to arrhythmogenesis.

In this presentation I will provide an overview of the developments that have occurred in the characterization of functional consequences of RYR2 mutations and their link to understanding arrhythmogenesis in CPVT patients. The presentation will provide a concise overview of the physiology of Ca2+ handling in the sarcoplasmic reticulum (SR) and its relevance to rhythm maintenance. Than I will provide a discussion on how mutations in RyR2 disrupt the Ca2+ handling system, leading to cardiac arrhythmias. Finally, I will address how the understanding of the pathophysiology of the disease may lead to novel therapeutic strategies.

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Integrins and vascular remodeling– Insights using atomic force microscopy

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The ability of vascular smooth muscle cells (VSMCs) to regulate vascular caliber is anchorage-dependent in that cells require physical attachment to the extracellular matrix (ECM) and to neighboring cells in order to perform the necessary mechanical work. Importantly, modulation of this vasoregulatory property of VSMCs would significantly impact autoregulation of tissue blood flow as well as control of peripheral vascular resistance. Integrin-mediated attachment of VSMCs to ECM proteins provides a site of attachment that physically creates a functional mechanical axis with the cytoskeleton for bidirectionally transmitting mechanical forces and generation of biochemical cell signals through inside-out and outside-in mechanisms.

In previous work, we have demonstrated that integrins play a role in modulating vascular tone and are important for regulation of arteriolar diameter and the vascular myogenic response supporting specific involvement in signaling related to contractile behavior. In other laboratories, biochemical evidence indicates that smooth muscle cell contraction is accompanied by rapid parallel remodeling of the actin cytoskeleton that occurs coincident with the development of tone. This remodeling is hypothesized to function to strengthen the contractile axis for transmission of force. Questions remain whether or not this type of actin remodeling characterizes the responses of VSMCs from resistance arterioles and if this type of remodeling process occurs concurrently with contractile changes in cell shape and whether it is synchronized with integrin adhesion. Enhanced integrin adhesion would be expected to also assist in bearing the increased mechanical loads associated with contractile activation. In these studies we have used atomic force microscopy (AFM) as a biomechanical method for real-time monitoring of cell contraction, cortical elasticity and adhesion to test the hypothesis that the integrins and their interactions with the ECM are enhanced and coordinated with contractile activation in VSMCs. The AFM allows this hypothesis to be biomechanically tested such that events related to contractile activation (cell movement), cytoskeletal remodeling (cell elasticity) and adhesion could be mechanically assessed and simultaneously measured. VSMCs were isolated from skeletal muscle arterioles and studied using AFM probes with tips that were bio-functionalized with ECM proteins providing a site of attachment that physically creates a functional mechanical axis with the cytoskeleton for bidirectionally transmitting mechanical forces and generation of biochemical cell signals through inside-out and outside-in mechanisms.
ent as an increased unbinding force and increased probability of binding between the AFM probe and the VSMC. These results support the hypothesis that control of VSMC adhesion to the ECM is dynamically coordinated with VSMC contractile activation and a change in cell elasticity. The parallel linking of integrin adhesion with contractile activation and cytoskeletal remodelling is potentially important to expanding our current framework of understanding of resistance artery function. Support to CAM NIH1P01HL095486.

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SA74
Apoptosis and spiral artery remodelling
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Important changes take place in the maternal uterine vasculature during the first few weeks of pregnancy resulting in increased blood flow to the developing fetus. Vascular endothelial and smooth muscle cells are lost from the spiral arteries and are replaced by fetal trophoblast cells. Failure of the vessels to remodel sufficiently in the first trimester is a common feature of pregnancy pathologies such as early pregnancy loss, intrauterine growth restriction and pre-eclampsia. There is evidence to suggest that some vascular changes occur prior to trophoblast invasion, however, in the absence of trophoblasts remodelling of the spiral arteries is greatly reduced. Until recently our knowledge of these events has been obtained from immunohistochemical studies which, although extremely useful, give little insight into the mechanisms involved. We have developed in vitro models including co-culture, time-lapse microscopy and spiral artery perfusion systems to investigate these events at a cellular and molecular level we are beginning to get a clearer picture of the regulation of spiral artery remodelling at the maternal-fetal interface. Trophoblasts synthesise and release a number of cytokines and growth factors including members of the tumour necrosis factor family. Our studies suggest that these factors may be important in regulating the remodelling process by inducing both endothelial and vascular smooth muscle cell apoptosis. More recently we have examined the role of maternal immune cells and in particular decidual natural killer cells in this complex process and have identified factors that they release that not only increase the motility of trophoblasts but also assist in remodelling by inducing vascular cell apoptosis. Studies into the aetiology of pre-eclampsia are hampered by the lack of an appropriate animal model and the inability to identify in the first trimester those individuals destined to develop pre-eclampsia. To address this we have used uterine artery Doppler ultrasound scanning to screen first trimester pregnancies in women attending clinics for surgical termination of pregnancy. Using this approach we have established significant differences in remodelling processes in pregnancies that are most and least likely to have developed pre-eclampsia.

The Wellcome Trust and the British Heart Foundation

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SA75
Cytoskeletal Mechanisms in Airway Smooth Muscle Remodelling
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Smooth muscle phenotype may be modulated in response to external conditions under physiologic and pathophysiologic conditions. Mechanical stress and humoral stimuli may activate signaling pathways that regulate changes in smooth muscle phenotype via cytoskeletal signaling pathways activated by integrin proteins. Integrin linked kinase (ILK) is a multidomain protein kinase that binds to the cytoplasmic domain of β integrins and forms a heterotrimERIC complex with the adaptor protein, PINCH, and with α-parvin: the ILK/PINCH/parvin (IPP) complex. Alpha-parvin binds to actin filaments; thus the IPP complex can link integrins to the actin cytoskeleton. The IPP complex is maintained as a stable complex in the cytoplasm of tracheal muscle tissues and is recruited to adhesion junctions in response to contractile stimulation, where it mediates ACh-induced actin polymerization (Zhang et al., J. Biol. Chem., 2007). In tracheal smooth muscle, ILK modulates the expression of smooth muscle specific marker proteins by regulating the activity of Akt; Akt activation suppresses SRF binding to the promoters of smooth muscle specific genes and suppresses the expression of smooth muscle marker proteins (Wu et al., Am J Physiol Lung Cell Mol Physiol, 2008). The effects of mechanical stimuli on tracheal smooth muscle phenotype were evaluated in vitro by suspending low or high loads (0.5 gm or 1 gm) from smooth muscle tissues, incubating the weighted tissues for 6 hours, and then measuring the expression of smooth muscle myosin heavy chain (SmMHC) and Akt activation. In muscles subjected to the higher load, expression of SmMHC increased and Akt activation was suppressed relative to tissues subjected to the lower load. The role of the IPP complex in regulating Akt activation and the expression of SmMHC protein in response to mechanical stimulation was evaluated. Mutant constructs for the PINCH LIM1-2 peptide, which inhibits recruitment of the IPP complex, kinase inactive ILK (ILK S343A), or kinase inactive Akt (T308A, S473A) were expressed in tracheal muscle tissues. All 3 mutants decreased Akt activation and increased expression of SmMHC protein of tissues incubated at low load but had little effect on SmMHC expression induced by high load. Thus, the localization of the IPP complex to integrin adhesion sites and activation of ILK and Akt suppressed the mechano-sensitivity of SmMHC expression, suggesting that integrin proteins are the primary sensors for mechanical signals that regulate smooth muscle phenotype. Stimulation of tissues with the inflammatory mediator IL-13 activated Akt and inhibited expression of SmMHC, opposing the effects of mechanical load. These effects of IL-13 were suppressed by inhibiting the recruitment of the IPP complex, indicating that the IPP complex recruitment is required for the phenotypic changes induced by IL-13. We conclude that mechanical stimulation maintains the differentiated state of airway smooth muscle via integrin-mediated signaling pathways, and that inflammatory stimuli may suppress the stimulatory effects of mechanical load on the expression of smooth muscle phenotype-specific contractile proteins.

Role of ion channels in smooth muscle remodeling

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The diversity of vascular smooth muscle cells (VSMCs) functions requires different cell phenotypes. For that reason VSMCs are not terminally differentiated, but retain a high degree of plasticity and are able to switch from a contractile to a proliferative and secretory phenotype. This process, known as phenotypic switch, is central to neointimal formation, which constitutes a common pathological lesion in diverse cardiovascular diseases, including coronary heart diseases, postangioplasty restenosis, and transplantation arteriopathy. Phenotypic modulation of VSMCs requires a dramatic change in gene expression profile. An important element in this change is the switch in ion transport mechanisms, as they represent a fundamental system required to redirect cell machinery to new functional tasks. The heterogeneity of ion channels expression pattern across different vascular beds has provided a large number of candidates implicated in the phenotypic switch, but only some of them have been found to be conserved in different VSMCs preparations.

In order to circumvent this limitation, we have obtained a global portrait of ion channel expression in contractile versus proliferating mouse femoral arteries VSMCs by using high-throughput real-time PCR. We analyzed the expression of 90 ion channel genes in two experimental paradigms: an in vivo model of endoluminal lesion and an in vitro model of cultured VSMCs obtained from explants. BPN mice (Jackson laboratories) were housed under temperature-controlled conditions (21°C) with free access to water and food. Endoluminal lesion of the common femoral artery was performed as previously described1 in mice anesthetized using isoflurane inhalation (0.5-1% at 2.5 l O2 min-1). To collect arterial samples mice were killed by decapitation after isofluorane anesthesia. All animal protocols were approved by our Institutional Care and Use Committee, and are in accordance with the European Community guiding principles in the care and use of animals.

Changes in mRNA expression showed a good correlation between the two proliferative models, with only two genes, Kv1.3 and Kvbeta2, increasing their expression upon proliferation. These mRNA changes translate into similar changes in protein expression levels in both proliferation models. While in contractile VSMCs Kv1.5-mediated currents are predominant, in proliferating VSMCs there is a net increase in the Kv1-mediated component of the Kv current due to the up-regulation of Kv1.3 channels2. Besides, functional studies demonstrate that the up-regulation of Kv1.3 currents in these cells is an essential component of their migratory and proliferative phenotype.

As the increased expression of Kv1.3 channels has also been described in proliferating VSMCs from human saphenous veins3, we explored whether this Kv1.3 up-regulation is a conserved landmark of VSMCs proliferation in different vascular beds, as this will point to these channels as good therapeutic targets to avoid unwanted VSMC remodelling. We have analyzed VSMCs from different vascular beds and different species, including human samples, and in all cases we found a predominant expression of Kv1.3 channels in the proliferative phenotype. Moreover, the selective blockade of Kv1.3 currents produced a significant decreased of VSMCs proliferation rate in all the preparations studied. Finally, we have been able to reproduce the pro-proliferative effect of Kv1.3 in a heterologous expression system. This system is becoming a useful tool as it provides a good preparation to explore the molecular determinants and the signalling cascade linking the functional expression of Kv1.3 channels to cell proliferation.


This work was supported by grants from the Spanish Ministerio de Sanidad, ISCIII (R006/009, Red Heracles), Ministerio de Ciencia y Tecnologia (BFU2007-61524 and BFU2010-15898) and Generalitat de Catalunya (CIDEM-VALTEC09-1-0042).

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Role of stretch in vascular smooth muscle remodelling

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Smooth muscle tissues exhibit a marked plasticity in their response to mechanical forces as well as to neural, endocrine and metabolic stimuli. This enables the tissues to rapidly adapt to differing functional demands. Adult smooth muscle cells are not terminally differentiated, and hence are able to revert to a synthetic phenotype with reduced contractility but high capacity for proliferation, migration and synthesis of extracellular matrix. In the vascular system, such phenotype shifts typically occur in response to endothelial injury, inflammation or lipid infiltration, leading to development of atherosclerotic plaques or of neointima formation following vascular surgery. Hypertension induces a different form of growth process, where cells are maintained in contractile phenotype and grow primarily by hypertrophy with much lower proliferation rates than in the synthetic phenotype. The signal mechanisms regulating growth processes must therefore be able to distinguish these different conditions, and much evidence indicates that stretch of the vascular wall has a key role, likely involving integrins and focal adhesion signalling as well as activation of G-protein coupled receptors. Overall protein synthesis and proliferation are stimulated by growth factor signalling and activation of the MAP kinase cascade, but is also rapidly activated by stretch. Stretch is furthermore able to increase the intracellular Ca2+ concentration, with downstream effects on gene expression depending on the mode of Ca2+ influx, via voltage-dependent and –independent pathways (Ren et al. 2010). In many vascular tissues, phenotype shift has been shown to be associated with a decrease of voltage-depend-
ent L-type Ca\(^{2+}\) channels and an increase of non-voltage dependent, particularly store-operated, channels (Kumar et al. 2006).

The synthesis of most contractile and cytoskeletal proteins, marking the contractile smooth muscle phenotype, is regulated by serum response factor (SRF) in concert with the coactivators myocardin and myocardin-related transcription factors (MRTFs). These factors bind to multiple conserved sites (CARG boxes) in the promoter regions of the smooth muscle genes. Growth factor stimulation causes displacement of myocardin from SRF and thus competes with differentiation. While myocardin is localised to the nucleus, MRTF is bound to monomeric G-actin in the cytoplasm and translocates to the nucleus when released from this binding. Polymerisation to filamentous F-actin reduces the cytoplasmic G-actin concentration and promotes nuclear translocation of MRTF. Stretch of vascular smooth muscle increases actin polymerisation via activation of RhoA and stimulates synthesis of smooth muscle proteins, thus stabilising the contractile phenotype. However, a further effector potentially regulated by RhoA is myocardin, which in contrast to smooth muscle markers is not dependent on SRF but is a transcriptional target of myocyte enhancer factor-2 (MEF2; Creemers et al. 2006). RhoA stimulation by membrane depolarization increases myocardin mRNA, which is mediated via activation of Rho-associated kinase and MEF2 transcription (Ren et al. 2010). Stretch increases myocardin mRNA as well and hence seems to stabilise the contractile phenotype via at least two Rho-dependent mechanisms, myocardin expression and MRTF translocation.

MEF2, in addition to its possible role in smooth muscle differentiation via Rho activation and voltage-dependent Ca\(^{2+}\) influx, is well established to be a mediator of growth factor signalling. Repression of MEF2 activity by binding to histone deacetylase 4 (HDAC4) is released by phosphorylation of HDAC4, requiring Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMK II; Li et al. 2010). This activation mechanism is linked to store-operated Ca\(^{2+}\) influx (Ren et al. 2010), and hence the balance between smooth muscle growth and differentiation is regulated by multiple interacting mechanisms involving stretch as well as growth factors and Ca\(^{2+}\) influx pathways. Integration of these pathways enables diverse phenotype regulation in response to external stimuli and muscle activity, including simultaneous growth and differentiation in stretch-induced hypertrophy.

Creemers EE et al. (2006). Development 133, 4245-56.

Supported by the Swedish Research Council

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A role for purinergic signalling in adult neurogenesis

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Neurogenesis proceeds in two major regions of the adult mammalian CNS. The subependymal zone (SEZ) at the lateral ventricles harbours stem cells that continuously provide new neurons for the olfactory bulb, whereas progenitors situated in the subgranular layer of the hippocampus (SGL) continuously produce new granule cells. Neurogenesis involves a variety of cellular events including cell proliferation, cell fate determination, migration, differentiation, integration and survival of young neurons. These processes are controlled by a symphony of cellular signals. We have investigated the functional role of nucleotides in the control of adult neurogenesis in the murine brain. Once released from cells to the extracellular milieu – nucleotides represent key mediators of cellular communication both in the CNS and in other tissues. They can act on a multiplicity of receptors (G protein-coupled P2Y and ionotropic P2X receptors) that differ regarding agonist specificity and the induced intracellular signal pathways. Nucleotide signalling is terminated or modulated by cell surface-located nucleotidase-hydrolyzing enzymes (ectonucleotidases).

We have previously shown that ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2), an enzyme that hydrolyzes extracellular nucleoside triphosphates to the respective nucleotide monophosphates, is specifically expressed by neural progenitors in the SEZ and SGL and that functional P2Y receptors are expressed in primary culture of adult neural stem cells (neurospheres or adherent cell cultures) (1-3). Furthermore both nucleotides and the growth factor EGF stimulate in vitro progenitor cell proliferation and migration and induced converging intracellular signalling pathways, implicating a possible role for nucleotides in adult neurogenesis (3-5).

Deletion of NTPDase2 should increase the extracellular concentrations of P2 receptor agonists such as ATP or UTP in the vicinity of the NTPDase2-depleted cells and thus enhance any nucleotide-mediated effect on neurogenesis. Proliferation of neural progenitors cells derived from NTPDase2 knockout mice and cultured as neurospheres in vitro was enhanced by a factor of two as compared to wild type controls. We then investigated potential in vivo effects of NTPDase2 deletion. Mice were subjected to time-controlled protocols of intraperitoneal BrdU application and the survival of BrdU-labelled cells was investigated in tissue sections two hours (proliferation and short term survival) and four weeks (long-term survival) after the end of BrdU application. As compared to wild type controls, progenitor cell proliferation was increased twofold in both the SEZ and the dentate gyrus in the NTPDase2 knockout animals. However, young neuron survival in the olfactory bulb and in the hippocampus was not significantly altered. These data suggest that NTPDase2 knockout, presumably associated with an increase in extracellular nucleotide concentrations in the neurogenic niches, enhanced progenitor cell proliferation. Nucleotides would, however, not support long term survival of young neurons. The data provide first in vivo evidence for a contribution of purinergic signalling to the control of adult neurogenesis.

Modulation of hippocampal synaptic plasticity by adenosine A2A receptors

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Inhibitory adenosine A1 receptors (A1Rs) are abundant throughout the brain, being present at pre-, post and non-synaptic sites to restrain neuronal excitability (e.g. Sebastião and Ribeiro, 2009). Excitatory actions of adenosine A2A receptors (A2ARs) in the hippocampus have been reported by us long ago but their scarce density in brain areas other than the striatum, namely in the hippocampus soon raised the hypothesis that the role of these receptors is fine tuning synapses rather than exerting a direct influence in synaptic transmission (see Sebastião and Ribeiro, 2009). Synapse fine tuning by A2ARs in the hippocampus has been recently extended to the tripartite synapse, including modulation of neurotransmitter transport in nerve endings and astrocytes as well as facilitation of responses to neurotransmitters and neuromodulators, with impact for synaptic plasticity, and I will refer to those studies.

Activation of adenosine A2ARs enhances extrasynaptic AMPA receptor mediated responses in the hippocampus, by means of a PKA-dependent mechanism; this correlates with increases in the surface expression of GluR1 subunits phosphorylated at the Ser 845 residue, as well as with enhancements in synaptic transmission and plasticity, suggesting that A2ARs adjust the availability of the extrasynaptic pool GluR1-containing AMPA receptors for synaptic insertion and consequent reinforcement of synaptic strength (Dias et al., 2010). A distinct way used by A2ARs to reinforce synaptic strength is to gate plasticity-related BDNF actions (Diógenes et al., 2011), through a mechanism that involves translocation of BDNF TrkB receptors to lipid rafts (Assaife-Lopes et al., 2010). By allowing BDNF-induced inhibition of cholinergic inputs to inhibitory interneurons (Fernandes et al., 2008), A2ARs may also contribute to shape excitatory transmission in the hippocampus.

A2ARs can interfere with the life-span of GABA at synapses, since they regulate the activity of GABA transporters (GATs). Indeed, GATs can be regulated by phosphorylation and we showed that GABA transport is facilitated by A2ARs in nerve endings (Cristovão-Ferreira et al., 2009). A2ARs facilitate transport of adenosine into nerve endings, limiting its availability to activate inhibitory adenosine A1 receptors (see Sebastião and Ribeiro, 2009), therefore contributing to a reduction of the adenosinergic inhibitory tonus at the synapses.

Results obtained from alert-behaving mice provided evidence for a direct and endogenous role of A2ARs in the potentiation of hippocampal synaptic responses evoked during the acquisition of an associative learning task, since both synaptic potentiation and concomitant associative learning were prevented upon A2AR blockade (Fontinha et al., 2009). In summary, A2ARs, by enhancing the shut down of the inhibition by GABA and adenosine, as well as by directly facilitating glutamatergic AMPA receptor-mediated responses and by gating BDNF actions, exacerbate the excitatory tonus at synapses, therefore contributing in multiple complementary ways for synaptic reinforcement and plasticity.

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zebrafish P2X4 receptor (3). It was found that four nucleotide binding segments (NBSs) situated at the interface of two neighbouring subunits act as agonist binding sites. Especially positively charged Lys and Arg residues are of great importance, but additional conserved and non-conserved amino acids also contribute to binding, gating or stabilization of the protein structure (4). High proton concentrations characteristically modify P2X3 receptor channels. They have a dual effect in that the current amplitude at low agonist concentrations is decreased (because of a decrease in the rate of desensitization), and increase at high agonist concentrations (because of a decrease in the rate of desensitization) (5). Replacement of histidine 206 but not histidine 45 by alanine abolished the pH-induced effects on hP2X3 receptors, locating the allosteric binding site for protons at the former amino acid. Eventually, P2X3 receptors may negatively interact with other pain sensing molecules such as the transient receptor potential vanilloid-1 (TRPV1) receptor, reacting to capsaicin, protons and heat. During the co-activation of these two receptors the current amplitude is lower than the sum of the individual currents caused by activation of each receptor alone (6). The inhibitory interaction did not depend on the holding potential, by the replacement of external Ca2+ by Ba2+, or when the buffering of intracellular Ca2+ was altered. However, the C-terminal truncation at Glu362 of P2X3 receptors abolished the TRPV1/P2X3 cross-talk. Co-immunoprecipitation studies with polyclonal antibodies generated against TRPV1 and P2X3 showed a visible signal in HEK293 cells transfected with them. Hence, these two pain-relevant receptors may interact with each other in an inhibitory manner probably by physical association established by a motif located at the C-terminal end of the P2X3 receptor distal to Glu362. In conclusion, P2X3 receptors are important pain sensing molecules activated by extracellular ATP and being regulated by acidic pH. They may interact with other pain-sensing receptors such as TRPV1 to protect an individual from overly strong pain. Information on the ligand binding site of P2X3 receptors may help to develop new antagonists with analgesic properties.


We are grateful for financial support to the Deutsche Forschungsgemeinschaft (Bonn)

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SA81

Role for ATP receptors in glia-neuron communication

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Communication between neuronal and glial cells is regarded to be very important for brain functions such as memory and cognition and many brain pathologies like ischemia, epilepsy and Alzheimer disease. Astrocytes enwrap neurons and therefore are exposed to various neurotransmitters spilled out of synaptic cleft. In response, astrocytes can release of gliotransmitters, such as D-serine, glutamate and ATP, and modulate synaptic strength and signalling within neuronal networks.

Extracellular ATP can acts as neurotransmitter mediating the excitatory synaptic transmission in the central nervous system. More importantly, ATP can also mediate the signal transfer between neuronal and glial circuits and within glial networks. ATP released from glial cells can modulate synaptic plasticity and development of neural cells and contribute to various pathological processes. These effects of ATP are mediated by ionotropic P2X and metabotropic P2Y receptors abundantly expressed in many types of neurons and glial cells.

There is growing evidence that release of gliotransmitters, including ATP, from the astrocytes shares the common mechanisms of vesicular neurotransmitter release, such as dependence on the proton gradient, SNARE proteins and intracellular Ca2+ elevation. Physiological role of ATP release from astrocytes was suggested by the data on participation of ATP in propagation of glial Ca2+-waves and significant contribution of purines to the astroglia-driven modulation of neuronal activity. Still, most of the evidence of Ca2+-dependent exocytosis of gliotransmitters has been provided by in vitro experiments using astrocytes in culture thus casting doubts on the functional importance of this mechanism in situ and in vivo. Traditionally, the major role in activation of Ca2+-dependent gliotransmitter release was suggested for the metabotropic P2Y receptors, abundantly expressed in astrocytes. The role for metabotropic Ca2+ signalling was questioned recently when experiments on genetically modified mice with altered InsP3/Ca2+ cascade found that neither enhancement not inhibition of astroglial metabotropic Ca2+ signalling affects synaptic transmission in hippocampus. So, the mechanism of glial exocytosis of ATP and other gliotransmitters and its importance for regulation of neuron signalling in situ and in vivo remain controversial.

The results of our recent experiments in neocortical astrocytes and neurons could help to resolve these controversies. Firstly, we have shown that cortical astrocytes express functional ionotropic receptors to ATP, composed mainly from P2X1 and P2X5 subunits. Astroglial P2X1/5 receptors exhibit very high affinity to ATP and, together with astroglial NMDA receptors, mediate fast glial synaptic currents (GSCs) triggered in the cortical astrocytes in response to stimulation of neuronal afferents. Astroglial P2X1/5 receptors have considerable Ca2+ permeability and their activation triggered robust transient Ca2+ signals in the cortical astrocytes. We have also found that maturation and ageing of the brain of mice (from 1 to 20 months) affected the purinergic signalling in cortical astrocytes: the density of P2X receptors and ATP-mediated component of Ca2+-signalling are smallest in young, maximal in adult and once more decrease in the aged mice.

Secondly, we have demonstrated that vesicular release of ATP from cortical astrocytes can be activated via various pathways including Ca2+-permeable ionotropic astroglial receptors or direct UV-uncaging of intracellular Ca2+. We have not observed release of ATP from astrocytes of dnSNARE transgenic mice in which the SNARE-dependent exocytosis was selectively impaired in astroglial cells. We have also found out that release of ATP from the neocortical astrocytes caused considerable decrease in the amplitude of both synaptic and tonic inhibitory currents in the cortical pyramidal neurons. This effect was mediated by phosphorylation of GABA A receptors activated by Ca2+-entry through the neuronal P2 purinoreceptors. Furthermore, modulation of neuronal inhibition by astrocyte-driven ATP affected the induction of long-term synaptic plasticity in the neocortex. Both release of ATP from astrocytes...
and its modulatory effects on synaptic transmission were eliminated in dnSNARE mice. These findings demonstrate an importance of SNARE complex-dependent exocytosis of ATP for glia-neuron interaction in the neocortex. Our results show a novel pathway of glia-neuron communication involving vesicular release of ATP from astrocytes and interaction between P2 and GABA receptors. Our data also imply that ATP-mediated communication between astrocytes and neurons in the neocortex undergoes remodeling during brain ageing and decrease in the ATP release from astrocytes may contribute to the age-related impairment of synaptic plasticity.

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SA82

Using optogenetics to study astrocytic purinergic signalling in the brainstem

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In 2005 Gourine et al. (Gourine et al., 2005) demonstrated that ATP is released at the ventral surface of the medulla oblongata during CO2-evoked activation of the central chemoreceptor area. It was also found that ATP release constituted an important initial event in central chemosensory transduction process. We hypothesised that ATP could be released from the local chemosensitive astrocytes and that these cells constitute an important link in this elusive mechanism. To study astrocyte activity in vivo and in vitro we expressed a genetically encoded Ca2+ indicator Case12 in ventral medullary astrocytes from Sprague Dawley or Wistar rats using viral vectors. Mild acidification in vivo (under α-chloralose anaesthesia) (100 mg kg-1 iv, supplemented with 20 mg kg-1 iv as required following femoral vein cannulation under 3% isoflurane induction) and in vitro triggered ATP-mediated Ca2+ waves in astrocytes located at the ventral edge of the medulla. Acidification-induced Ca2+ responses were abolished in the presence of bafloynacine (a blocker of vesicular pumps) and brefeldine A (blocker of vesicular transport) indicating that ATP is released from a vesicular pool. In vitro, ATP receptor antagonists reduced electrophysiological and Ca2+ responses of the local population of chemosensitive neurones (of the retrotrapezoid nucleus, RTN) to changes in pH, suggesting that ATP-mediated signalling plays a key role in central chemosensory transduction. In order to selectively stimulate astrocytes we expressed in these cells a light-sensitive non-selective cation channel ChR2. Stimulation of transduced astrocytes with blue light triggered Ca2+ elevations and release of ATP as measured by luciferase assay. Light activation of astrocytes in the central chemosensitive area (overlapping with RTN) led to activation of the respiratory activity in vivo and this effect could be blocked by MRS2179 an antagonist with preferential tropism to P2Y1 and P2X1 receptors. These experiments demonstrated the involvement of astroglial purinergic signalling mechanisms in one of the most fundamental physiological processes in the CNS(Gourine et al., 2010). To further study astrocyte-to-neuron signalling, we have generated vectors to express light-sensitive G-protein coupled receptors (OptoAlpha1- and OptoBeta2)(Airan et al., 2009) selectively in astrocytes using vectors with enhanced GFAP promoter(Liu et al., 2008). Both OptoAlpha1 and OptoBeta2 vectors were studied in vitro and we have verified that OptoAlpha1 signals via PLC and IP3 while OptoBeta2 signals via adenyly cyclase. Light activation of OptoAlpha1—expressing astrocytes in the rostral-ventro-lateral medulla (RVLM) evoked powerful pressor responses and increases in renal sympathetic nerve activity. Similar responses are evoked following activation of ChR2 expressed in RVLM glia. The signalling mechanisms used by astrocytes in RVLM are currently under investigation. In summary, optogenetic approaches allow interrogation of the intermechanisms underlying astrocyte-neuronal signalling in vitro and in vivo and demonstrate that purinergic signalling plays an important role in astrocyte-neuronal communication in brainstem autonomic structures.


Financial support: British Heart Foundation and Wellcome Trust.

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SA83

Regulation of Sodium Transport and Pro-inflammatory Cytokine Secretion by the Stress-activated Metabolic Sensor AMP-activated Protein Kinase in Human Bronchial Epithelial Cells

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The stress-activated metabolic sensor AMP-activated protein kinase (AMPK) inhibits both the cystic fibrosis transmembrane conductance regulator (CFTR) and the epithelial Na+ channel (ENaC) in polarized epithelial cell lines (1-4). In addition, AMPK subcellular localization is disrupted and AMPK activity is up-regulated in primary polarized cystic fibrosis (CF) human bronchial epithelial (HBE) cells relative to non-CF HBE cells, an effect that may help tamp down the secretion of pro-inflammatory cytokines (5). We have tested in primary CF versus non-CF HBE cells the effects of the mechanistically distinct pharmacological AMPK activators metformin and 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR) on various functionally relevant parameters that contribute to CF lung disease: ENaC and CFTR-dependent short-circuit currents (Isc), airway surface liquid (ASL) reabsorption rates, and pro-inflammatory cytokine secretion (6). As compared with controls, AMPK activation following overnight treatment with either metformin (2.5 mM) or AICAR (1 mM) substantially inhibited ENaC dependent Isc in both cell types as well as CFTR-dependent Isc in non-CF cells. ASL reabsorption rates over time were measured by live-cell confocal microscopy following apical addition of PBS containing Texas Red-dextran.
ALSL heights were significantly greater following AICAR and metformin treatment at 60 min in both CF and non-CF cells relative to controls, suggesting that AMPK-dependent inhibition of ENaC in airway cells slows apical fluid reabsorption. Extending our previous results obtained using immortalized CF versus non-CF cell lines, we also found that both metformin and AICAR decreased the apical inhibition of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 6, and interleukin 8 in primary CF and non-CF HBE cells, both in presence and absence of prior stimulation with lipopolysaccharide (LPS). Finally, much lower concentrations of metformin (0.03 – 1 mM) given over longer time periods, corresponding more closely to levels achieved therapeutically in vivo, were sufficient to cause inhibitions of ENaC-dependent currents and pro-inflammatory cytokine levels in CF HBE cells. The inhibition of ENaC and pro-inflammatory cytokine secretion by AMPK may be mediated in part by AMPK-dependent inhibition of IkB kinase-β, an upstream regulator of the NF-κB pathway. These findings suggest that novel therapies to activate AMPK in the CF airway may be of benefit both by blunting excessively high ENaC activity and thus ASL hyperabsorption and by reducing excessive airway inflammation, which are both major contributors to CF lung disease.


This work was supported by the NIH/NIDDK and Cystic Fibrosis Foundation.

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SA84

Role of cytokines in controlling gene expression and sodium transport in airway and alveolar epithelial cells; implications for cystic fibrosis and pulmonary edema

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Sodium absorption by the amiloride-sensitive epithelial sodium channel (ENaC) is the main driving force involved in lung edema clearance. Recently, the regulation of this sodium transport mechanism in injured lung is evaluated [1]. There is accumulating evidence that the expression level of ENaC is modulated in injured lungs. In a chronic Pseudomonas aeruginosa lung infection model, we observed a distinctive expression profile for the three subunits (α, β and γENaC) [2]. All the subunits showed an increased expression at 24h post infection followed by a decrease on day 3. In a model of ischemia-reperfusion lung injury (single lung transplantation), we also observed a decreased expression of ENaC mRNA in the transplanted lungs [3]. Furthermore, we found that alveolar liquid clearance was significantly lower in transplanted lungs and that alveolar liquid clearance could not be stimulated by an adrenergic agonist. Although the mechanism involved in this downregulation of ENaC expression is not known at the moment, we propose that inflammatory mediators could potentially be involved. In fact, TNFα a pro-inflammatory cytokine involved in these lung injury models decreases the expression of the α, β and γENaC mRNA subunits and had a major impact on the transepithelial current generated by the alveolar epithelial cells [4]. Our recent data also shows that other inflammatory molecules such as LPS can inhibit ENaC expression and activity in alveolar epithelial cells. We were able to show that this effect of LPS involves the release of ATP from alveolar epithelial cells and is most likely associated with a stimulation of the purinergic receptor (P2Y2) [5]. Can this effect of inflammatory molecules on ENaC expression and activity be inhibited by anti-inflammatory treatment? Because the synthetic steroid dexamethasone has been shown to upregulate ENaC mRNA expression [6], and is well-known to downregulate pro-inflammatory genes, we tested if it could alleviate the effect of TNF on ENaC expression and activity. In co-treatment with TNF, we found that dexamethasone reversed the inhibitory effect of TNF and upregulated α, β and γENaC mRNA expression [7]. In addition to its effect on αENaC gene expression, dexamethasone was able to maintain amiloride-sensitive current as well as the liquid clearance abilities of TNF-treated cells within the normal range. All these results suggest that dexamethasone alleviates the downregulation of ENaC expression and activity in TNF-treated alveolar epithelial cells. Overall, these data suggest that ENaC expression in injured lung can be modulated by inflammatory molecules. The impact of these changes in expression on the evolution of lung injury remains to be determined.


Supported by grants from CIHR and Cystic Fibrosis Canada
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**SA85**

**Inflammation, infection and airway glucose homeostasis**

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In humans, the normal glucose concentration of airway secretions is around 0.4mM; 12.5 times lower than plasma concentrations [1]. Airway glucose concentrations are increased by inflammation. We have found elevated luminal glucose concentrations in upper airways of people with viral rhinitis [2] and in lower airways of people with asthma, chronic obstructive pulmonary disease and cystic fibrosis [1]. Airway glucose concentrations are also increased by hyperglycaemia. The effect of inflammation and hyperglycaemia is synergistic, with further elevation of airway glucose in patients with cystic fibrosis who also have diabetes [1].

Airway luminal glucose concentrations are the net effect of transepithelial movement of glucose into the lumen and removal of glucose from the lumen. In polarised monolayers of human immortalised bronchial (H441) cells, L glucose (a non-transportable, non-metabolisable analogue) added to the basolateral compartment appeared in the apical compartment at concentrations inversely proportional to transepithelial electrical resistance [3]. Glucose thus moves across the epithelium down its concentration gradient to an extent determined by properties of intracellular junctions. In similar experiments, less D glucose (transportable and metabolisable) than L glucose appeared in the apical compartment, but apical D glucose concentrations increased in the presence of apical or basolateral phloretin. This indicates that apical and basolateral facilitated glucose transporters (GLUTs) restrict glucose appearance in the apical compartment. Phloretin also restricted apical appearance of lactate, indicating that airway epithelial cell glucose uptake through GLUTs is driven by a concentration gradient generated by intracellular glucose metabolism. A role for GLUTs in epithelial glucose homeostasis is supported by identification of GLUT2 protein in H441 cells and human bronchial epithelium on biopsy [4] and of GLUT10 in primary cultured human airway epithelial cells [5].

Inflammation disrupts epithelial glucose homeostasis. In H441 monolayers, pro-inflammatory cytokines reduce epithelial resistance, which is associated with increased transepithelial glucose flux [3]. By contrast, pro-inflammatory cytokines increased epithelial GLUT2 and GLUT10 protein expression and increased apical glucose uptake, possibly as a compensatory mechanism. Elevated glucose concentrations in inflamed airways therefore appear to be attributable to increased paracellular movement into airways secretions, overwhelming compensatory upregulation of glucose transport. Other investigators have shown structural abnormalities of tight junctions in inflamed airway epithelium, reproducible in vitro by exposure of cultured, airway epithelial cells to proinflammatory cytokines, which increase paracellular permeability to hydrophilic solutes.

Elevated airway luminal glucose concentrations are associated with infection. In intubated patients on our intensive care unit, elevated airway glucose concentrations were associated with increased risk of acquiring respiratory methicillin resistant Staphylococcus aureus infection [6]. Glucose at concentrations found in lung secretions had a dose-dependent effect on growth of S. aureus and Pseudomonas aeruginosa in laboratory culture [7]. In a cultured airway epithelial cell model, increasing basolateral glucose concentrations increased apical glucose concentrations and caused a dose-dependent increase in growth of S. aureus at the epithelial cell surface. Other studies in this model have indicated that glucose increases adherence of S. aureus to the apical epithelial surface, possibly by enhancing integrin expression. In support of our findings, Pezzulo and colleagues found that increased glucose concentration in airway surface liquid augments growth of Ps. aeruginosa in vitro and in the lungs of hyperglycemic mice in vivo [5]. By contrast, hyperglycemia had no effect on intrapulmonary bacterial growth of a Ps. aeruginosa mutant that is unable to utilise glucose as a carbon source [5].

In summary, epithelial mechanisms normally maintain low glucose concentrations in airways secretions. This appears to be important for defence of the lung against infection. Inflammation and hyperglycaemia elevate glucose concentrations in lung secretions, promoting growth and pathogenicity of respiratory pathogens. Infection may lead to further inflammation with enhanced glucose leak fuelling the vicious cycle of infection and inflammation in chronic lung disease. Maintenance of low airway glucose concentrations represents a new therapeutic target in the field of lung infection.


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SA86

Molecular Mechanisms of Intestinal Epithelial Tight Junction Regulation

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Epithelial barrier dysfunction contributes to progression of intestinal and systemic disease. However, there is a fundamental gap that separates clinicopathologic significance from molecular understanding of the mechanisms responsible for barrier regulation. The major components of the tight junction, which forms the paracellular barrier, have been identified over the past two decades and fall into three major groups: scaffold proteins, e.g. ZO-1; transmembrane regulatory proteins, e.g. occludin; and pore-forming proteins, e.g. claudins. How these proteins interact to regulate the barrier is incompletely understood. Thus, tight junction biology is at a crossroads that requires a transition from protein discovery to identification of the means by which these proteins interact to regulate function. To better understand these interactions, we have recently applied in vitro and in vivo fluorescence recovery after photobleaching (FRAP) analyses with in vitro protein binding studies and measures of tight junction barrier function. The data indicate that modulation of tight junction protein intermolecular interactions is a mechanism of barrier regulation, and implicate ZO-1 as a critical intermediate in these molecular events. Moreover, disruption of these regulatory processes can reverse cytokine-induced barrier dysfunction. Thus, in addition to providing novel insight into the mechanisms of physiological and pathophysiological barrier regulation, these data provide new opportunities for therapeutic intervention.

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SA87

Role of wtCFTR in Airway Epithelial Inflammatory Responses to Bacterial Exoproducts

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A hallmark of cystic fibrosis (CF) lung pathology is the accumulation of bacteria, usually including Pseudomonas aeruginosa, and the triggering of an exuberant innate immune response, i.e., increased production of proinflammatory cytokines and chemokines and resulting recruitment of neutrophils to fight the infection. These events lead to characteristic damage to and remodeling of lung tissue characteristic of the disease. It has been proposed that this so-called hyperinflammatory response in CF results from a defect in the epithelial cells resulting in altered inflammatory signaling and increased release of proinflammatory mediators. This presentation will first summarize previous work from this lab in which this hypothesis was tested and discarded. The second part of the talk will present an alternative model based on recent experiments that indicate that an important aspect of wtCFTR’s role in controlling innate immune response is through its role as a cAMP/PKA-activated anion channel.

Using an adenovirus to express either wtCFTR or ΔF508CFTR in human CF airway epithelial cells in culture, we found that there was no role for CFTR in proinflammatory NF-κB or Ca signaling or in IL8 secretory responses to either Pseudomonas aeruginosa or flagellin, the key activator of toll-like receptor 5 in the epithelial cells. Expression of wtCFTR or ΔF508CFTR also had no effect on cytosolic or organelle redox or on the unfolded protein-stress response of the endoplasmic reticulum. It is proposed instead that the most important aspect of the wtCFTR in the innate immune response is its “classical” function as an anion channel. Transepithelial electrophysiology showed that wtCFTR is activated by both bacterial exoproducts (flagellin, pyocyanin, homoserine lactone) and also by proinflammatory mediators (IL1β, TNFα). It is expected that the presence of Pseudomonas aeruginosa in the airways will trigger secretion of proinflammatory mediators, and both the bacteria and the cytokines and chemokines will increase wtCFTR-dependent chloride and bicarbonate secretion and osmotically obliged fluid into the airway surface liquid. This fluid secretion will help flush the bacteria from the airways in nonCF but will be missing in CF, leading to accumulation of bacteria and increased proinflammatory response.

An unexplained aspect of this proposal is that wtCFTR is activated by cAMP/PKA, while none of the bacterial or proinflammatory mediators has been thought to activate this pathway. For example, the quorum-sensing molecule N-3-oxododecanoyl homoserine lactone (3OC12-HSL or C12) of Pseudomonas aeruginosa use to control expression of both virulence factors and biofilm in the airways has been shown to activate Ca signaling in several cell types, but not cAMP. Recent experiments on cultured human nonCF and CF cells in vitro using transepithelial and patch clamp electrophysiology and wide field, confocal and TIRF imaging methods show that C12 activates cAMP signaling through a store-operated cyclase model in which C12 activates IP3 receptors, which then leak Ca from the endoplasmic reticulum into the cytosol. The loss of Ca from the ER activates the ER-resident, Ca-sensitive protein regulator stim1, which migrates to the plasma membrane and activates a still to be identified adenylate cyclase in the plasma membrane to produce cAMP and activate CFTR. It seems likely that many bacterial and proinflammatory mediators trigger wtCFTR using a similar activation of cAMP in addition to their better known effects on NF-κB/p38 and Ca signaling.

Funding: NIH, Cystic Fibrosis Foundation, Cystic Fibrosis Research, Inc.

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SA88

Prenatal Determinants of Children’s Health

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Fetal development is largely dependent on the mother. However, pregnancy maintenance and consequently fetal development are highly vulnerable and sensitive to disruption, triggered by the macro- or microenvironment. High stress perception is becoming the ‘epidemic’ of the 21st century, as identified by a recent study carried out by the World Health Organisation in Northern European countries. Also, women
CRH, Urocortins and inflammation in human placenta


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Corticotrophin releasing hormone (CRH) is recognized as the hypothalamic neuropeptide that regulates the hypothalamic-pituitary-adrenal (HPA) axis in mammals, orchestrating neuroendocrine, autonomic and immunologic responses to stress. During pregnancy placenta represents the main source of CRH, acting via autocrine, paracrine and endocrine mechanisms, plays a critical role in the maintenance of pregnancy and regulates neuroendocrine and inflammatory events leading to physiological parturition and its premature onset (1). Neuroendocrine and immune systems interact at various levels. Interestingly, CRH serves a pro-inflammatory function by activating the immune/inflammatory response in several conditions (2). Indeed at the maternal–fetal interface, trophoblast cells produce pro-inflammatory cytokines in response to physiological and pathological conditions (4) and recent evidences have shown that CRH enhances LPS-induced pro-inflammatory cytokine expression, such as TNF-α and IL-8, in human trophoblast cells, through activation of p38/MAPK signalling and TLR-4 expression (3).

CRH exerts its biological effects by binding two G protein-coupled receptors named CRHR1 and CRHR2, both expressed by utero-placental tissues, with a prevalent affinity for CRHR1. Since antalarmin, the CRHR1 antagonist, has shown to suppress CRH effects on peripheral inflammation in non-gestational tissues, it is believed that CRHR1 may represent the main receptor mediating the pro-inflammatory effects of CRH (4). Recently, an increasing interest has been developed for the urocortins (Ucn, Ucn2, Ucn3) that belong to the CRH family neuropeptides and exert complimentary or sometimes contrasting actions to fine-tune CRH biological effects. Urocortins bind the CRH receptors with different affinity, in fact Ucn has approximately the same affinity for both receptors, whereas Ucn2 and Ucn3 bind only CRHR2 (1).

Ucn is expressed by intrauterine tissues (human placenta, decidua, and fetal membranes) throughout pregnancy and maternal plasma concentrations remain constant until term and increase significantly during term and preterm labour. Moreover, since Ucn levels in fetal circulation are higher than in maternal plasma, while Ucn mRNA expression doesn’t change between labouring and non-labouring placenta, a fetal source of the peptide has been hypothesized at term and preterm parturition (1). Similarly to CRH, Ucn acts as an endogenous immunomodulatory factor, but showing predominant anti-inflammatory effects and immune tolerance maintenance (2). Indeed, our group has recently demonstrated that Ucn modulates LPS-induced secretion of the pro-inflammatory TNF-α and increases the basal secretion of anti-inflammatory cytokines (IL-4, IL-10) from trophoblast cultures through CRHR2, suggesting that Ucn may modulate the placental inflammatory responses leading to physiological and pathological parturition (4).

Ucn2 and Ucn3 are expressed in early and late gestational tissues (human placenta, decidua, and fetal membranes) and mRNA levels are increased at lower oxygen tensions, but their role in the mechanism of term and preterm delivery remains unknown (6). With respect to their involvement on inflammatory pathways, some studies suggest that Ucn2 exerts mainly pro-inflammatory activity while Ucn3 may regulate anti-inflammatory effects in non-gestational tissues, although binding the same receptor (6). Indeed, recent in vitro data obtained by our group have shown that Ucn2 increases the pro-inflammatory while Ucn3 the anti-inflammatory cytokines secretion via CRHR2 in trophoblast cultures (unpublished work).

Finally, we have recently found that intrauterine inflammatory/infecative pathways such as chorioamnionitis associated with preterm delivery activate placental CRH/Ucn pathways in vivo (7). In fact, trophoblast samples collected from preterm deliveries associated with chorioamnionitis have shown up-regulation of CRH, Ucn2 and CRHR1 genes and down-regulation of Ucn, Ucn3 and CRHR2 genes in comparison to preterm deliveries not associated with chorioamnionitis. Furthermore, these changes have been reproduced in vitro by treating placental trophoblast with LPS, suggesting their potential importance in infection-mediated preterm labour.

In conclusion, CRH peptides and receptors modulate inflammatory responses in utero-placental tissues and their placental expression is activated when inflammatory/infecative processes occur, suggesting their role as important mediators of the interactions between neuroendocrine and immune systems leading to parturition and its premature onset.


288P
Maternal neuroendocrine-immune responses to stress in early pregnancy

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Stress during pregnancy has profound and long term detrimental consequences on maternal and fetal health, including on the maintenance of gestation itself. However, while intrauterine mechanisms underlying adverse effects on the developing fetus are becoming clear, the maternal neuroendocrine responses that mediate stress perception and signal to the periphery are often not reported. Many neuroendocrine hormones respond to stress and affect the feto-maternal interface, including the pregnancy-protective cytokine balance and cytokines act on the brain and pituitary to disrupt neuroendocrine secretion; e.g. mimicking infection with lipopolysaccharide (LPS) is a well-known stressor. Generally, stress robustly increases hormonal secretion from the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic system in pregnancy. However, the role of hormones such as glucocorticoids in stress-induced pregnancy failure is unclear. For example, evidence does not consistently show that elevated circulating glucocorticoids accompany pregnancy failure, so any role for them must depend upon local mechanisms in the feto-maternal interface such as the 11beta hydroxysteroid dehydrogenase enzymes that regulate local glucocorticoid concentration. In the absence of obvious maternal HPA axis signals mediating pregnancy failure, we investigated what other neuroendocrine signals link maternal stress perception with stress-induced peripheral effects using a pregnant mouse model. Various stressors (e.g. sound stress, hunger or immune stress) decrease secretion of key pregnancy hormones such as progesterone. One major neuroendocrine hormone system is involved in driving high progesterone secretion in rodent models and humans: anterior pituitary prolactin, whose secretion is elevated from early gestation. As well as enhancing progesterone secretion prolactin also directly facilitates implantation in the decidua and is an immune regulator. We have shown that unlike in virgins, stress strongly decreases prolactin secretion, so stress-induced lack of prolactin may compromise pregnancy maintenance. Indeed preliminary data in women indicates that low circulating prolactin correlates with pregnancy failure (Douglas 2010), and the literature shows that lack of prolactin or progesterone in early pregnancy leads to pregnancy failure, adverse fetal programming and suboptimal maternal behavior (Larsen and Grattan 2010; Pincus et al 2010).

We have investigated hypothalamic control of prolactin secretion in early pregnancy. Dopamine is the main neuroendocrine hormone controlling pituitary lactotrophs, and typically strongly inhibits lactotroph prolactin secretion. In early pregnancy dopamine control of prolactin is attenuated, allowing the increasing basal prolactin secretion. However, when the mother is exposed to stress there is hyperactivity of the neuroendocrine tuberoinfundibular dopamine (TIDA) neurones located in the arcuate nucleus and increased dopamine release in the median eminence, explaining the stress-inhibited prolactin secretion. Evidence shows that this response is similar regardless of the stressor used and provides a direct explanation for how stress can rapidly elicit detrimental effects on the important maternal hormonal mechanisms sustaining pregnancy.

Other hypothalamic neuroendocrine neurones additionally respond to the same stressors and some of these neuronal populations also control pituitary prolactin secretion; for example oxytocin and thyroid releasing hormone from paraventricular paraventricular nucleus neurones are prolactin releasing factors. In pregnancy responses of neurones in this region to some stressors (such as peripheral injection of LPS) are attenuated, suggesting reduced drive of the prolactin releasing factors in addition to the increased dopaminergic inhibition. LPS induces increased circulating cytokines which signal to the hypothalamus by driving prostaglandin secretion from blood vessel walls in the brain ependyma and activate brainstem neurones. The responses of neurones in the afferent stress-responsive noradrenergic brainstem pathways (such as the nucleus tractus solitarius) that project to the arcuate nucleus are enhanced in pregnancy, perhaps contributing to hyperactivation of the TIDA neurones. Therefore, there are multi-level adaptations in the maternal brain that exhibit altered responsiveness to stress that may result in attenuated pregnancy hormone secretion.

In conclusion, maternal neuroendocrine hormone systems play a prominent role in mediating the adverse consequences that occur when a pregnant mother is exposed to stress. The resulting lack of key pregnancy hormones such as prolactin and progesterone in the susceptible peri-implantation period inevitably causes an inhospitable intrauterine environment that risks ongoing pregnancy maintenance.

Douglas AJ. Front Neuroendocrinol 2010; 31: 359-376
Larsen CM, Grattan DR. Endocrinology 2010; 151: 3805-3814.
AJD was a member of the EU 6th Framework Network of Excellence on Embryo Implantation Control (EMBIC) and was funded by The Wellcome Trust, The MRC and The Society for Endocrinology.

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Stress, CRH and Cytokines in Childhood/Adolescence and Concurrent or Later Disorders

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In chronic stress, the reproductive axis is inhibited at all levels by various components of the activated hypothalamic-pituitary-adrenal (HPA) axis. CRH suppresses the secretion of GnRH either directly or indirectly, by stimulating the arcuate nucleus POMC peptide-secreting neurons. Glucocorticoids also exert an inhibitory effect on the GnRH neuron, the pituitary gonadotroph, and the gonads, and render target tissues of gonadal steroids resistant to these hormones. During inflammatory stress, the elevated concentrations of cytokines also result in suppression of reproductive function via inhibition of both GnRH pulsatile secretion from the hypothalamus and ovarian/testicular steroidogenesis.

Suppression of gonadal function secondary to chronic stress-related activation of the HPA axis has been demonstrated in highly trained runners of both sexes and ballet dancers. These subjects display elevated concentrations of serum cortisol and plasma ACTH in the evening, increased 24-hour urinary free cortisol excretion, and diminished ACTH responses to exogenous CRH administration. Males have low LH and testosterone concentrations, and females have amenorrhea. Interestingly, obligate athletes develop withdrawal symptoms and signs following discontinuation of their exercise routine, which may reflect withdrawal from the daily exercise-induced elevation of opioid peptides and stimulation of the mesocorticolimbic system. Hypothalamic oligo-amenorrhea, improper folliculogenesis, oligo-anovulation, corpus luteum insufficiency and hypofertility have been associated with chronic stress. Defective blastocyst implantation, miscarriages, and premature labor and delivery can also occur in stressful pregnancies. From the side of the embryo and fetus, stress during gestation can lead to intrauterine size restriction and prenatal programming for a stressful extra-uterine environment. This entails acquisition of epigenetic vulnerability to later stress hyper-responsive and metabolic syndrome manifestations. Very early stress related to assisted reproductive technologies also entails later risks, such as metabolic syndrome manifestations, for the pregnancy product.

The interaction between CRH and the hypothalamic-pituitary-gonadal axis is bi-directional, given that estrogen increases CRH gene expression via estrogen-response elements in the promoter region of the CRH gene. Therefore, the CRH gene is an important target of gonadal steroids and a potential mediator of sex-related differences in the stress-response and the activity of the HPA axis. Also, the dependence of the stress system upon estrogen explains estrogen withdrawal-related mood disorders, such as luteal phase dysphoric syndrome, postpartum blues and depression and climacteric depression.

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Research Symposia

SA91

SA92

Early life stress and reproductive dysfunction

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Adverse early environments can have a profound and lifelong influence on stress reactivity. Exposure of neonatal rats to stress, e.g. an immunological challenge of lipopolysaccharide (LPS), programs long-term changes in hypothalamo-pituitary-adrenal activity with increases in basal corticosterone pulse frequency and amplitude as well as marked increases in stress-induced corticosterone release in adulthood. This neonatal challenge also impacts on the hypothalamo-pituitary-gonadal (HPG) axis, resulting in delayed puberty and disruption to ovarian cyclicity which persisted into adulthood. The up-regulation of ovarian low-affinity receptor of nerve growth factor (p75NGFR), a key marker of raised sympathetic tone, may account for the observed increased thickness of the theca interna and reduced follicle reserve. The down-regulation of hypothalamic kisspeptin, identified as an essential gatekeeper of pubertal onset and gonadotrophic hormone secretion, could provide a mechanism for the observed delay of puberty and may be the first manifestation of more long-lasting changes to the HPG axis, namely disturbed ovarian cycles and sensitisation to stress-induced suppression of the GnRH pulse generator in adulthood. In the adult, considerable progress has been made concerning the mechanisms underlying corticotrophin-releasing factor (CRF)-induced suppression of GnRH pulse generator activity, including novel sites of action within key brain regions commonly associated with stress responsiveness. There is a differential involvement of CRF receptor type 1 and 2 in stress-induced suppression of the GnRH pulse generator, which is both stressor and brain loci specific. Moreover, the observed CRF or stress-induced down regulation of kisspeptin and its receptor (Kiss1r) expression in hypothalamic regions critical for the control of LH secretion suggests a key role for the integration of CRF and kisspeptin signalling in the regulation of the GnRH pulse generator. We have recently shown a novel role for CRF in the timing of puberty and the up-regulation of amygdaloid CRF with delayed puberty in neonatal-LPS treated rats suggests this limbic brain structure may contribute to the developmental cues that drive and modulate the kisspeptin system critical for reproduction.

Welcome Trust and BBSRC

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SA93

The Effects of Chronic Sustained and Intermittent Hypoxia on Muscle Physiology

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Skeletal muscles, including the striated muscles of breathing, have a remarkable capacity for remodelling as evident in various physiological and pathophysiological settings. Hypoxia -
a dominant feature of respiratory system malfunction – can drive phenotypic change in physiological systems including the respiratory control system with potential ‘adaptive’ or ‘maladaptive’ consequences for respiratory homeostasis. Despite the clinical relevance however, surprisingly little is known about the effects of chronic hypoxia on respiratory muscle physiology. We are exploring the effects of long-term hypoxia on respiratory muscle form, function and control in translational animal models of chronic sustained hypoxia (CSH) – a feature of chronic lung disease such as COPD, and chronic intermittent hypoxia (CIH) – a dominant feature of sleep-disordered breathing.

In adult male Wistar rats, we examined the effects of CSH (ambient pressure = 380mmHg for 1-6 weeks) [1] and CIH (20 cycles of hypoxia [5 or 10%] per hour; 8 hours a day for 1-2 weeks) on respiratory pump and upper airway muscle contractile and endurance properties, fibre type and size, oxidative capacity, relative area of fibres expressing sarco/endoplasmic reticulum calcium ATPase (SERCA2), and Na+K+ ATPase pump content. Moreover, we tested the hypotheses that NO is critically involved in CIH-induced respiratory muscle ‘adaptation’ whereas oxidative stress is implicated in CIH-induced respiratory muscle ‘maladaptation’.

The major findings of our studies are: 1) CSH improves diaphragm (but not sternohyoïd) muscle endurance; 2) CSH-induced muscle plasticity is time- and intensity-dependent and differentially expressed in respiratory muscles; 3) CSH causes diaphragm muscle fibre atrophy but does not alter fibre areal density; 4) CSH does not alter respiratory muscle oxidative capacity; 5) CSH does not increase the relative area of diaphragmatic fibres expressing SERCA2 protein; 6) CSH increases diaphragm Na+K+ pump content; 7) Chronic NO blockade attenuates CH-induced increases in Na+K+ pump content and prevents CH-induced functional remodelling in the diaphragm; 8) CIH increases diaphragm muscle fatigue; 9) CIH-induced muscle plasticity is time- and intensity-dependent; 10) CIH causes slow-to-fast fibre transition; 11) CIH does not affect respiratory muscle oxidative and glycolytic capacity; 12) CIH increases the relative area of diaphragmatic fibres expressing SERCA1 protein after 7 but not 14 days; 13) CIH does not affect respiratory muscle Na+K+ pump content; 14) Chronic antioxidant treatments prevent CIH-induced functional remodelling in the diaphragm.

To summarize, chronic hypoxia drives structural and functional remodelling in respiratory muscle. Sustained hypoxia is associated with NO-dependent ‘adaptation’ in diaphragm increasing Na+K+ pump content concomitant with increased muscle endurance. Conversely, intermittent hypoxia increases rat diaphragm fatigue, most likely due to increased oxidative stress as antioxidant treatment (N-acetyl cysteine and Tempol) or NADPH-oxidase inhibition prevents CIH-induced diaphragm dysfunction. We conclude that hypoxia-induced muscle plasticity is dependent upon the duration, intensity and pattern of hypoxic exposure and is differentially expressed in muscles with complementary function (i.e. airway dilator muscles vs. diaphragm). In separate studies we have established that hypoxic remodelling in respiratory muscle is dependent on age and sex. Our studies are providing novel insight into mechanisms involved in chronic hypoxia-induced respiratory muscle remodelling. The results may have relevance to respiratory disorders characterized by chronic hypoxia such as COPD and sleep apnoea where respiratory muscle remodelling is known to occur.


Supported by the Health Research Board Ireland.

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Chronic obstructive pulmonary disease as a systemic disease: a skeletal muscle perspective

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Peripheral skeletal muscle dysfunction is considered to be one of the primary determinants of disability in chronic obstructive pulmonary disease (COPD). Furthermore, the poor relationship between exercise capacity and lung function impairment in COPD suggests exercise intolerance in this patient group is not limited by pulmonary ventilation. This stand-point is supported by the observations that improvements in dyspnoea and exercise capacity that result from lung directed therapies are slow relative to those seen following exercise training mediated pulmonary rehabilitation.

Unlike normal healthy ageing where the loss of Type II muscle fibres predominates, COPD is characterised by the preferential loss of type I muscle fibres and a reduction in the maximal activity of several mitochondrial enzymes. It is unclear whether these responses are attributable to inactivity induced deconditioning rather than COPD per se. As might be expected, there is a reduction in mitochondrial ATP generation and greater reliance on non-mitochondrial energy production during exercise in COPD patients. Skeletal muscle adenine nucleotide loss is associated with fatigue during high intensity exercise in healthy volunteers, and reflects the inability of muscle ATP production to match the ATP demand of contraction. We have demonstrated that significant adenine nucleotide loss occurs in the skeletal muscles of COPD patients during exercise at considerably lower absolute workloads to those seen in healthy volunteers (2). Furthermore, efforts to reduce the magnitude of metabolic stress at the onset of exercise by pharmacologically activating the pyruvate dehydrogenase complex immediately prior to exercise, using a pyruvate dehydrogenase kinase inhibitor, reduced blood lactate and ammonia (a sensitive marker of adenine nucleotide loss) accumulation during exercise and improved maximal exercise performance in COPD patients (3). It is perhaps not surprising therefore that endurance exercise rehabilitation is effective at improving exercise capacity in COPD.

COPD patients have a lower muscle mass and strength compared to age and sex matched controls, and both are predictors of mortality, disability and healthcare utilisation in COPD, independent of lung function impairment (4, 5). Nonetheless, the aetiology of this phenotype is unknown. Several studies have shown functional benefits from resistance training in COPD. However, whilst resistance exercise training promotes muscle mass restoration after disuse in young (6) and older (7) people (albeit to a lesser extent in the latter), the impact of resistance training on muscle mass restoration in COPD, and the mechanisms therein, is less clear cut. This is at least partly attributable to progress in COPD being hampered by a lack of exercise intervention studies documenting temporal changes in genes and proteins thought to regulate muscle mass, and the dovetailing these observations with sensitive measures of muscle mass and muscle protein synthesis and breakdown. These are important generic issues because the choice of likely
therapeutic approaches in COPD will depend upon the outcome of such studies.


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SA95

Respiratory muscle function and training in patients with COPD

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The impact of chronic obstructive pulmonary disease (COPD) upon respiratory mechanics was described elegantly in the 1940s by Rahn and coworkers (Rahn et al., 1946). Thereafter, the mechanical repercussions of COPD were largely overlooked until a Renaissance of interest in the 1980s focussed attention upon the repercussions of COPD for the respiratory muscles. By the 1990s, a clear understanding had emerged that expiratory flow limitation, hyperinflation and dyspnoea were interlinked phenomena that contributed to exercise intolerance (O’Donnell, 2001). The influence of hyperinflation upon the work of breathing is profound, especially during exercise, when end expiratory lung volume increases dynamically as part of a strategy to maximise expiratory flow generating capacity. Whilst this strategy is effective in mitigating the influence of expiratory flow limitation upon minute ventilation, it does so at great cost to the inspiratory muscles, which must overcome both greater elastance, and the effects of intrinsic positive end expiratory pressure.

In the late 1990s, evidence also emerged that the increased inspiratory work imposed by COPD induces considerable remodelling of the diaphragm. Not only is there an increase in the proportion of fatigue resistant type I muscle fibres (Levine et al., 1997), but oxidative enzyme activities and capillarity also increase. These adaptations are consistent with a shift towards an endurance-trained phenotype, which has led to the perception that the diaphragms of patients with COPD are well adapted to chronic loading. This assumption is supported by the observation that patients with COPD showed no evidence of low-frequency diaphragm fatigue following treadmill exercise to the limit of tolerance.

However, in recent years, it has been recognised that the diaphragm remodelling of COPD is imperfect. Inspiratory muscle weakness is a prominent feature of COPD (Decramer, 2001), but like so much of the pathophysiology of COPD, its aetiology is complex. Weakness has two primary sources, both of which contribute to impaired function, 1) muscle fibre myopathy due to systemic manifestations of COPD, corticosteroids and changes in physical activity patterns; 2) functional weakening due to the interaction of hyperinflation and the pressure-volume relationship. As explained above, these decrements in the capacity of the inspiratory muscles to deliver ventilation are paralleled by a considerable increase in the work of breathing due to hyperinflation. Furthermore, during exercise, inefficiencies of breathing pattern, and the early onset of metabolic acidosis, also serve to increase the ventilatory requirement of exercise, exacerbating the demands placed upon the inspiratory muscles. Thus, patients with COPD experience a “double-whammy” of an increase in the demand for inspiratory muscle work, and a reduction in the capacity to meet that demand. This mismatch has serious implications for dyspnoea and exercise tolerance.

The functional imbalance within the respiratory pump of patients with COPD makes the respiratory muscles an obvious therapeutic target. A number of recent systematic reviews and meta-analyses support a beneficial influence of inspiratory muscle training (IMT) upon inspiratory muscle function, dyspnoea and exercise tolerance (Gosselink et al., 2011). There is evidence that IMT elicits beneficial structural adaptation within the accessory inspiratory musculature of patients with COPD, as well as diaphragm hypertrophy in healthy individuals. Furthermore, evidence is also emerging from healthy individuals that IMT improves exercise tolerance, at least in part, via an increase in the activation threshold of the inspiratory muscle metaboreflex (McConnell & Lomax, 2006). When activated by high intensity inspiratory muscle work, this reflex has been shown to induce limb muscle vasoconstriction, and impaired fatigue resistance (McConnell & Lomax, 2006). Following IMT, the delayed onset of the reflex, preserves limb blood flow, enhances oxygen delivery, and exercise tolerance (Romer et al., 2002; McConnell & Lomax, 2006). Replication of these observations in patients with COPD is awaited, but there is every reason to believe that IMT elicits similar changes to metaboreflex activation.


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SA96

Effects of iron on the pulmonary circulation: a therapeutic role in COPD?

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Chronic occlusive pulmonary disease (COPD) is a common, progressive condition associated with considerable symptom burden for patients, and cost implications for society. Cur-
rent therapies are of limited benefit. This talk will highlight the rationale behind the novel use of an established therapy, intravenous iron.

The pulmonary circulation as a therapeutic target Current COPD therapies have focused on the airways. Consideration of the pulmonary circulation remains a neglected area, despite the fact that in COPD pulmonary hypertension and increased pulmonary vascular resistance are associated with increased hospitalisation (Kessler, Faller et al. 1999) and mortality (Burrows, Kettle et al. 1972) and decreased exercise capacity (Sims, Margolis et al. 2009). Furthermore pulmonary hypertension with a mean pulmonary vascular pressure >20mmHg is common at around 1/3 at rest (Weitzenblum, Hirth et al. 1981; Doia, Nakano et al. 2003) and 2/3 on exercise (Oswald-Mammoser, Apprill et al. 1991; Christensen, Ryg et al. 2004), and pulmonary vascular resistance is almost universally raised (Burrows, Kettle et al. 1972). Therapies to optimise the pulmonary circulation may therefore reduce morbidity and mortality in COPD.

Candidates for pulmonary circulation optimisation As yet, drugs used with success in idiopathic pulmonary hypertension, such as the endothelin-1 antagonist, Bosentan, have not been effective in pulmonary hypertension of COPD (Stolz, Rasch et al. 2008). Furthermore, Bosentan worsens oxygenation in COPD, due to worsening ventilation: perfusion matching (Stolz, Rasch et al. 2008). In hypoxic COPD patients long term oxygen therapy is the only therapy that has been shown to reduce mortality (1980; 1981) and ameliorate pulmonary hypertension (1981; Weitzenblum, Sautegeau et al. 1985) and raised pulmonary vascular resistance(1980; 1981; Weitzenblum, Sautegeau et al. 1985). A limitation of long term oxygen therapy is that it is only associated with mild improvements in pulmonary haemodynamics in COPD (1980; 1981). This is because there is no reversal of pulmonary vascular remodeling (Willkinson, Langhorne et al. 1988), which contributes to pulmonary hypertension through narrowing of small and precapillary arteries (Peinado, Pizarro et al. 2008). This difference may be in part because long term oxygen therapy is non-continuous, tendency to be used for 15 hours a day. Alternative agents are therefore urgently sought. We propose iron could be an alternative effective therapy.

Rationale for the use of intravenous iron We have recently demonstrated for the first time that the normal pulmonary vascular pressure rise in response to hypoxia is iron dependent; first, intravenous iron blunts the usual acute hypoxic pulmonary vasoconstriction after 8 hours (Smith, Balanos et al. 2008), and one week’s (Smith, Talbot et al. 2009) hypoxic exposure in healthy volunteers. Secondly, lowering iron availability using iron chelation over 8 hours (Smith, Balanos et al. 2008), or venesection of high altitude residents with chronic mountain sickness (Smith, Talbot et al. 2009) augments hypoxic pulmonary vasoconstriction. These effects of iron are plausible, as there is a strong biochemical rationale for interplay between oxygen and iron, potentially acting via the hypoxia inducible factor (HIF) pathway. HIF is a family of transcription factors that controls hundreds of genes involved in oxygen sensing and delivery. One of its effects is development of pulmonary hypertension, as observed by our group in patients with the rare genetic disorder, Chuvash polycythemia, in which HIF-mediated gene activation is pathologically increased (Smith, Brooks et al. 2006). Control of HIF is by degradation of the HIF-alpha subunit by prolyl hydroxylases. This is an iron dependent process (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Yu, White et al. 2001), hence iron deficiency may up regulate but iron supplementation down regulate HIF activity.

The future challenges Future challenges include optimal assessment techniques in COPD patients, in whom non-invasive pulmonary vascular measures are suboptimal and elucidating molecular mechanisms by which iron affects the pulmonary circulation.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

SA97

When obesity and COPD collide: Physiological and clinical consequences

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The prevalence of both chronic obstructive pulmonary disease (COPD) and obesity is increasing steadily throughout the western world. Several recent studies demonstrate that the prevalence of obesity is greater among patients with COPD than in the general population. A recent population study found that in Canada, the prevalence of obesity in COPD was as high as 25% and greater than the non-COPD population (17%) (1). Unlike the general population where obesity prevalence is escalating, it has remained stable over a 13 year observation period in COPD. Increased activity limitation and healthcare utilization were found much more commonly in obese COPD patients compared with non-obese COPD patients.

The presence of obesity has paradoxically been shown to convey a survival advantage in patients with more severe COPD but extreme obesity in COPD has been associated with increased respiratory-related mortality (2). Obese subjects with COPD demonstrate reduced exercise capacity as measured by six-minute walk distance but not as measured by cycle endurance tests (3-6). To begin to reconcile these apparent contradictions, a better understanding is needed of the interactions between COPD, obesity, activity limitation and respiratory symptoms.

A recent study has shown that increasing BMI has profound effects on resting pulmonary function measurements in patients with airway obstruction (7). Thus, residual volume and functional residual capacity decreased exponentially as BMI increased in COPD. The volume reducing effects of increased BMI are seen even in the overweight range and it is noteworthy that existing predictive equations for lung volume do not account for this important effect.

Given the known restrictive ventilatory deficit associated with obesity and the propensity for increased airway closure and expiratory flow limitation at the lower operating volumes, one would anticipate that the combination of obesity and COPD would have added deleterious effects with respect to dyspnea and exercise intolerance. However, when one compares ventilatory constraints during cycle exercise (which unloads the excess body mass) in obese and lean COPD patients, matched for FEV1, there is no clear disadvantage seen in the former (3-6).

Obese COPD patients demonstrate reduced exercise capacity as measured by six-minute walk distance but not as measured by cycle endurance tests (3-6). Exercise endurance time, measured during constant work rate exercise testing, has been shown to be similar in obese and normal weight COPD patients despite increased metabolic and ventilatory requirements associated with excessive weight (3-6).

We have postulated that the volume reduction effects of obesity in COPD and the recruitment of a higher resting inspiratory capacity convey a mechanical advantage that counter-balances the known restrictive effects and increased elastic loading of obesity (4-6). Obesity causes lung deflation of similar magnitude to that achieved during pharmacological or surgical volume lung volume reduction.
A recent mechanical study has shown that in patients with moderate COPD who have mild obesity, static elastic recoil pressure of the lungs and intra-abdominal pressures are significantly increased compared with lean COPD patients with similar airway obstruction (6). Mean expiratory flow rates during exercise were not diminished despite lower operating lung volumes in the obese COPD group, possibly because of the increased driving pressure for flow. However, we were unable to show any significant advantage with respect to diaphragmatic function in the obese COPD patient compared to the normal weight control. Respiratory muscle performance and recruitment patterns throughout exercise were similar in obese and lean COPD groups (6).

These studies collectively indicate that abnormalities in dynamic ventilatory mechanics and the associated respiratory discomfort are not appreciably greater in obese COPD patients than in lean controls, at least during cycle exercise. During weight-bearing exercise (treadmill walking), we anticipate that the higher metabolic and ventilatory requirements of this task will force an earlier onset of critical respiratory mechanical constraints in obese COPD patients than during weight-supported cycle exercise at comparable power outputs. However, it remains to be seen whether the long-held belief that “respiratory factors are the proximate cause of activity limitation during daily activities in patients with combined COPD and obesity” is actually true.

Vozoris NT, O’Donnell DE. Prevalence, risk factors and health outcomes in an obese population cohort with COPD. (manuscript in review)


Canadian Thoracic Society.

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