Immunolocalization of the zinc transporter hZTL1 at the apical membrane of human jejunum and Caco-2 cells

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We previously reported the cloning of an intestinal zinc transporter hZTL1 which, when expressed in human intestinal Caco-2 cells with a C-terminal Myc epitope tag, was located at the apical membrane (Cragg et al. 2002). Here, we confirm the localization of hZTL1 in Caco-2 cells and report the location of hZTL1 in sections of human jejunum using an hZTL1 antipeptide antibody.

Rabbit anti-hZTL1 antibody was raised against a synthetic peptide corresponding to amino acids 190–211 of hZTL1 (ILSSPSKRCQKGTLLG1YSPEGT) and was affinity purified. Human jejunum was obtained from cadaver donors with appropriate ethics committee approval. Frozen sections (7 μm) were fixed for 30 min on ice with 4% (w/v) paraformaldehyde in PBS. Caco-2 cells, grown for 14 days on polycarbonate filters, were fixed with 100% methanol for 5 min at room temperature. Sections or cells were permeabilized in 0.1% (v/v) Triton X-100 in PBS and incubated in blocking solution (5% w/v BSA, 10% (v/v) goat serum, 0.1% Triton X-100 in PBS). After incubation overnight at 4°C with anti-hZTL1 antibody (1:100), slides were washed in PBS and samples were then incubated with FITC-conjugated goat anti-rabbit IgG (1:500) for 1 h at room temperature. Caco-2 cells were subsequently treated with propidium iodide to reveal nuclear staining or with a chromogenic alkaline phosphatase stain. Sections or filters were mounted in fluorescence mounting medium under a sealed coverslip and visualized using a Leica confocal microscope.

Specific FITC staining was localized at the apical membrane of human jejunum and was concentrated towards the villus tip. In Caco-2 cells, staining was observed exclusively at the apical membrane and co-localized with alkaline phosphatase activity. These data are consistent with the established location of hZTL1 in Caco-2 cells using the Myc-tagged construct (Cragg et al. 2002).

Comparison of the cDNA sequences of hZTL1 and ZnT5 (Kambe et al. 2002) reveals that they are splice variants of the same gene. The antibody used in the study reported here corresponds to a region common to both hZTL1 and ZnT5. We have previously reported that the ZnT5 variant, shown to localize to intracellular vesicles in pancreas (Kambe et al. 2002), is not detected in Caco-2 cells by RT-PCR (Russi et al. 2003). The absence of a vesicular staining pattern in either human intestine or Caco-2 cells reported here is consistent with the expression of only hZTL1 in intestine and adds to the evidence that hZTL1/ZnT5 splice variants are expressed in a tissue-specific manner.

Russi RM et al. (2003). J Physiol (in Press) P.

This work was supported by BBSRC grant 13D/11012

All procedures accord with current UK legislation and the use of human tissue was covered by ethics committee approval.

Identification of the placental Cu oxidase as eleutherin, a hephaestin homologue

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The mechanism of iron release from the placenta is not well understood. We have previously identified a copper oxidase that appears to be intimately involved in the process and have shown that it was regulated by copper and by iron levels in placental cells (BeWo) (Danzeisen et al. 2002). Recently, we identified sequences in the database with a high degree of homology to both hephaestin and serum ceruloplasmin (www.ncbi.nlm.nih.gov accession numbers XP-100543 and XP-146812). Here, we demonstrate, using reverse transcriptase–polymerase chain reaction (PCR), siRNA, Western blotting and real-time PCR, that this gene codes for the placental Cu oxidase.

BeWo cells were grown in Ham’s F12 supplemented with 10% fetal bovine serum with or without antibiotics as appropriate. Cells were grown to approximately 80% confluence. For siRNA experiments, the cells were treated according to methods provided on the Ambion web site (www.ambion.com) and using templates designed according to the manufacturer’s instructions (Ambion Silencer® siRNA Construction Guide). Cells were transfected with the siRNA using siPortAmine (Ambion) according to the manufacturer’s instructions after optimisation for BeWo cells. Following transfection, cells were scraped and solubilised in running buffer before being separated on 7.5% SDS–PAGE gels. Oxidase protein was visualised using anti-ceruloplasmin antibody (Danzesien et al. 2002). Real-time PCR was performed using the TaqMan system (Perkin Elmer).

As would be expected, the sequences showed a putative membrane-spanning sequence and multi-copper binding site. Reverse transcriptase–PCR reactions gave a single band of the predicted size, which, when sequenced, matched the gene in the data base. We tested the hypothesis that the mRNA coded for the oxidase protein by using siRNA followed by immunoblotting. Transfected the cells with increasing concentrations of siRNA resulted in a dose-dependent decrease in oxidase protein. This is direct evidence that the gene codes for the placental oxidase.

We have previously shown that the oxidase protein and activity levels are regulated by copper and iron. Here, we demonstrate, using real time PCR, that mRNA levels are decreased by treatment with a copper chelator, but are not altered by iron chelators. We propose calling this member of the Cu oxidase family ‘eleutherin’ after Eleutheria, the sister of Hephaestos and the goddess of midwives.


This work was funded by SEERAD, EUFPV, and the International Copper Association.
PKG II-dependent inhibition of an Fe\textsuperscript{2+}-evoked electrogenic transport pathway by cGMP in human intestinal Caco-2 epithelia

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Apical exposure to Fe\textsuperscript{2+} stimulates a pH-dependent inward short circuit current (I\textsubscript{SC}) in Caco-2 epithelia (Scott et al. 2002). This transport process is consistent with H\textsuperscript{+}-coupled apical entry of Fe\textsuperscript{2+} via DMT-1 (Gunshin et al. 1997). The present study set out to investigate intracellular regulation of Fe\textsuperscript{2+}-evoked electrogenic transport by cGMP.

I\textsubscript{SC} determinations were made on voltage-clamped Caco-2 epithelia, grown on permeable supports (Anotec, Nunc). Cells were bathed with isotonic mannitol–Hepes buffer (37°C; pH 7.4). At the onset of the experiment, apical pH was adjusted to 6.0. Iron ascorbate (1:10 molar ratio) was applied apically. The cGMP analogue 8-Br cGMP (100 μM) was applied to the apical bathing medium for 20 min prior to Fe\textsuperscript{2+} exposure. In experiments investigating the effects of staurosporine (0.5 μM), the PKA inhibitor H-89 (50 μM), and the PKG II inhibitor Rp-8-pCPT-cGMPs (20 μM), these were applied 30 min prior to 8-Br cGMP.

Exposure to 8-Br cGMP inhibited Fe\textsuperscript{2+}-induced inward I\textsubscript{SC} to levels not distinguishable from zero at all Fe\textsuperscript{2+} concentrations tested (25–1000 μM). Pre-incubation with staurosporine reversed cGMP-induced inhibition of Fe\textsuperscript{2+}-evoked I\textsubscript{SC} and stimulated (P < 0.05, ANOVA) an additional inward I\textsubscript{SC} (control V\textsubscript{max} = 1.06 ± 0.05 μA cm\textsuperscript{-2} (9); stauro + cGMP V\textsubscript{max} = 1.71 ± 0.12 μA cm\textsuperscript{-2} (5) (means ± S.E.M. (n))). Staurosporine alone stimulated the Fe\textsuperscript{2+}-induced I\textsubscript{SC} with V\textsubscript{max} rising (P < 0.001 vs. control) to 2.45 ± 0.40 μA cm\textsuperscript{-2} (4). Rp-8-pCPT-cGMPs also abolished the inhibitory action of 8-Br cGMP on Fe\textsuperscript{2+}-induced I\textsubscript{SC} (control V\textsubscript{max} = 1.05 ± 0.15 μA cm\textsuperscript{-2} (4); Rp-8-pCPT-cGMPs + cGMP V\textsubscript{max} = 0.99 ± 0.04 μA cm\textsuperscript{-2} (4)). As with staurosporine, Rp-8-pCPT-cGMPs alone stimulated the Fe\textsuperscript{2+}-induced I\textsubscript{SC}, V\textsubscript{max} rising (P < 0.01) to 1.99 ± 0.21 μA cm\textsuperscript{-2} (4). H89 had no effect on the actions of 8Br-cGMP or on the Fe\textsuperscript{2+}-evoked I\textsubscript{SC}.

These data identify an intracellular regulatory mechanism for an Fe\textsuperscript{2+}-evoked electrogenic transport pathway in Caco-2 epithelia. Regulation by cGMP was inhibitory and was dependent upon PKG II with no discernible involvement of PKA. The stimulatory actions of both staurosporine and Rp-8-pCPT-cGMPs when applied alone suggest that there was baseline inhibitory PKG II activity in these cells. To our knowledge, this is the first reported involvement of cGMP/PKG II-dependent regulation of an Fe\textsuperscript{2+}-evoked transport pathway.


D.A.S. was supported by a BBSRC PhD studentship.

Iron and zinc differentially regulate Fe\textsuperscript{2+}- and Zn\textsuperscript{2+}-induced electrogenic transport pathways in human intestinal Caco-2 epithelia

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We have previously reported on the stimulation of pH-dependent electrogenic transport pathways following exposure to apical Fe\textsuperscript{2+} and Zn\textsuperscript{2+} in Caco-2 epithelia (Scott et al. 2002). The present study set out to establish the effects of chronic Fe\textsuperscript{2+} and Zn\textsuperscript{2+} loading on Fe\textsuperscript{2+} depletion on these transport routes.

Short circuit current (I\textsubscript{SC}) determinations were made on voltage-clamped Caco-2 epithelia, grown on permeable supports (Anotec, Nunc). Cells were bathed with isotonic mannitol/Hepes buffer (37°C; pH 7.4). At the onset of the experiment, apical pH was adjusted to 6.0. Iron ascorbate (1:10 molar ratio) or zinc histidine (1:5) was applied apically. For Fe\textsuperscript{2+} or Zn\textsuperscript{2+} loading, 100 μM of either Fe\textsuperscript{2+} or Zn\textsuperscript{2+} was added to culture medium for 24 h prior to experimentation. For Fe\textsuperscript{2+} depletion, 100 μM desferrioxamine was added to culture medium 40 h prior to experimentation. All conditions were accompanied by time-matched vehicle controls from the same cell batch.

Under Fe\textsuperscript{2+}-depleted conditions, the Fe\textsuperscript{2+}-induced inward I\textsubscript{SC} was enhanced with V\textsubscript{max} rising significantly (P < 0.01, Student’s unpaired t test, means ± S.E.M. (n)) from 0.97 ± 0.12 (5) to 2.04 ± 0.18 μA cm\textsuperscript{-2} (5). In contrast, Fe\textsuperscript{2+} depletion resulted in a reduction in the Zn\textsuperscript{2+}-induced I\textsubscript{SC}, V\textsubscript{max} falling (P < 0.02) from 1.25 ± 0.07 (4) to 0.95 ± 0.05 μA cm\textsuperscript{-2} (4). Fe\textsuperscript{2+} loading reduced the Fe\textsuperscript{2+}-evoked I\textsubscript{SC} at all Fe\textsuperscript{2+} concentrations tested (1–500 μM; P < 0.05–0.001) with transport failing to display saturation. However, Fe\textsuperscript{2+} loading had no effect on the Zn\textsuperscript{2+}-induced I\textsubscript{SC}. Zn\textsuperscript{2+} loading enhanced the Fe\textsuperscript{2+}-induced I\textsubscript{SC} by over 300% with V\textsubscript{max} rising (P < 0.001) from 0.89 ± 0.13 (5) to 3.4 ± 0.19 μA cm\textsuperscript{-2}. In striking contrast, Zn\textsuperscript{2+} loading reversed (P = 0.001) the inward I\textsubscript{SC} observed under control conditions (V\textsubscript{max} = 0.77 ± 0.12 μA cm\textsuperscript{-2} (5)) to a significant (P < 0.05) saturable outward I\textsubscript{SC} with a V\textsubscript{max} of 1.11 ± 0.27 μA cm\textsuperscript{-2}.

These data demonstrate that Fe\textsuperscript{2+} and Zn\textsuperscript{2+}-evoked electrogenic transport pathways are differentially affected by altering the exposure levels to these metals. This provides evidence that some or all of the transporters involved in these pathways are regulated by both Fe\textsuperscript{2+} and Zn\textsuperscript{2+}. The present data are consistent with previously published data demonstrating regulation of DMT-1 expression by Fe\textsuperscript{2+} and Zn\textsuperscript{2+} (Yamaji et al. 2001). However, it does not support the view that Fe\textsuperscript{2+} and Zn\textsuperscript{2+} are transported by a common pathway.


D.A.S. was supported by a BBSRC PhD studentship.
Expression of divalent metal transporter DMT1 is coordinated during development and spermatogenesis in rat testis


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Iron is essential to fertility as it is required for germ cell division, differentiation and metabolism. DMT1 is expressed in the apical membranes of duodenal enterocytes and is responsible for the non-transferrin-mediated absorption of dietary iron. DMT1 is also expressed in vesicular membranes and participates in transferrin-mediated iron acquisition by cells. It is highly expressed in the kidney and is thought to play a role in renal iron handling. The aim of this study was to determine the pattern of DMT1 expression in the rat testis.

Peroxidase immunohistochemistry using an affinity-purified anti-DMT1 polyclonal antibody targeted to recognise all DMT1 isoforms (Ferguson et al. 2001) was used to study the cellular distribution of DMT1. Western analysis was used to quantify the levels of DMT1 in the testis of humanely killed 5-, 15-, 25-, 35-day-old and adult rats.

DMT1 immunoreactive species were detected throughout development in the seminiferous tubules but not in the interstitium. In the 5- and 15-day-old rats, immunostaining was equally widespread throughout each tubule and could be localised to the cytoplasm of the Sertoli cells. This was the case in the 25- and 35-day-old animals but more punctate areas could be detected.

In the adult rat immunostaining was specific to each of the 14 stages of the cycle of the seminiferous epithelium and as a result tubules appeared heterogeneous, unlike in the immature rat. Staining could be localised to the nuclei and cytoplasm of both spermatogonia and mature spermatozoa, suggesting that these cells have a high requirement for iron as they prepare to leave the seminiferous tubules.

Western analysis detected an immunoreactive protein band of around 60 kDa in the membrane fractions at all ages tested. At 15, 25 and 35 days and in the adult rat a second higher weight band was detected of around 70 kDa, revealing expression of a different isoform at these ages. Deglycosylation experiments with N-glycosidase showed that the major DMT1 isoform present (55 kDa) was different from that in the kidney (50 kDa).

The expression profile of DMT1 in the development of the rat testis was found to be cell specific and highly co-ordinated to the spermatogenetic cycle. This suggests an important role for DMT1 in spermatogenesis and implies that the germ cells have a need for a precisely timed supply of iron. This stage-specific nature of expression means that DMT1 plays a central role in male fertility. This may be applicable when considering conditions of abnormal iron regulation.


This work was funded by the BBSRC, The Royal Society and The Welcome Trust

All procedures accord with current UK legislation.

Site-directed mutagenesis investigations of the substrate-binding site of the rabbit proton-coupled peptide transporter PepT1 expressed in Xenopus oocytes

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The proton-coupled di- and tri-peptide transporter isofrom PepT1 has an exceptionally wide substrate range, leading to interest in understanding the substrate-binding site (see Meredith & Boyd, 2000 for a review). Here we report the results of site-directed mutagenesis experiments to test whether particular highly conserved residues play a predictable role in PepT1 function, with the aim of further refining our substrate template model (Bailey et al. 2000).

Selected rabbit PepT1 residues were mutated using a PCR-based protocol (Quikchange, Stratagene) and confirmed by DNA sequencing. Uptake experiments into PepT1-expressing Xenopus laevis oocytes were performed as previously described (Meredith et al. 2000). Data are means ± S.E.M.; n = 5 oocytes.

His121 has been proposed as playing a role in the binding of the side chain of the second residue in a dipeptide, with the protonated form (HisH+) donating a proton to the COO- of acidic residues (Chen et al. 2000). The H121A, H121N and H121F PepT1 mutations were virtually unaffected, as might be predicted for uptake of a neutral substrate (Fig. 1). Arg282 has been predicted from computer modelling to be involved in regulating translocation of substrates through PepT1, although the Vmax of R282A-hPepT1 was only reduced 15 % (Bolger et al. 1998). Indeed, as can be seen in Fig. 1, even after swapping the wild-type positively charged residue for a negative glutamate, R282E-PepT1 could still perform transport. Finally, Glu594 has been proposed as the binding site for the amino terminus NH3+ group of substrates (Meredith et al. 2000). Conservative substitution of this residue for negatively charged Asp (E594D) abolished uptake, suggesting that simply the presence of a negatively charged amino acid residue at this point in the sequence is insufficient for functional protein.

These preliminary results confirm and extend existing studies implicating His121 and Glu594 in the functioning of the PepT1 transport protein. The role of Arg282 is currently less clear.
Further studies will be required to elucidate the full roles of these residues in PepT1 in substrate binding and translocation.


This work is generously funded by the Wellcome Trust.

All procedures accord with current UK legislation.

C8

Altered affinity for cationic amino acid transport through system y+L following forskolin treatment of cultured BeWo cells

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The human placental trophoblast tumor cell line BeWo can be induced to syncytialise following forskolin. During a microarray expression study of forskolin-regulated genes in these cells (Kudo et al. 2002) we noted striking and reciprocal changes (up-regulation, down-regulation respectively) in the levels of mRNA encoding the amino acid transporters y+LAT-2 and y+LAT-1. These membrane proteins are the two isoforms of the catalytic light chains of the y+L amino acid transport system. We have therefore studied transport through system y+L in these cells with and without 36 h pre-treatment with forskolin to see whether there are alterations in transport function associated with the altered gene expression.

Table 1. y+L mediated lysine flux (V/Vi)

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<th>Control</th>
<th>+Forskolin</th>
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<tr>
<td>+Lys (10 µM)</td>
<td>0.60</td>
<td>0.42</td>
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<tr>
<td>+Lys (100 µM)</td>
<td>0.26</td>
<td>0.14</td>
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Table 2. y+L mediated arginine flux (V/Vi)

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<tr>
<th></th>
<th>Control</th>
<th>+Forskolin</th>
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<tbody>
<tr>
<td>+Arg (10 µM)</td>
<td>0.32</td>
<td>0.21</td>
</tr>
<tr>
<td>+Arg (100 µM)</td>
<td>0.13</td>
<td>0.05</td>
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Because we were interested in determining both affinity and velocity for different known system y+L substrates we used a mixture of two cationic amino acids (lysine, arginine) as substrates, each at low concentration (1 µM) with a modified protocol for determining amino acid influx (Kudo & Boyd, 2002) under initial rate conditions (3 min, 37 °C) using double label ([14C]arginine, L-[3H]lysine). We observe high affinity inhibition of more than 95% of total cationic amino acid influx by 10 mM glutamine in both control and forskolin (100 µM) treated cells (K is 90 µM in control and 30 µM in treated cells). Since in the absence of sodium (choline substituting), total cationic amino acid transport fluxes are substantially unaltered whereas the affinity for inhibition by glutamine is very markedly reduced, we conclude that at the low substrate concentration used in this study virtually all of the flux of cationic amino acids into these cells is through system y+L (in contrast to the conclusion of Way et al. 1998). We find (Tables 1 and 2) that cells treated with forskolin have a higher affinity for inhibition (V) of system y+L by either amino acid than do control (vehicle-only) cells. Taken together with the array data this indicates that, in mammalian cells at 37 °C, y+LAT1 has a lower affinity than does y+LAT2 for cationic amino acids (compatible with findings made in the Xenopus oocyte expression system at room temperature; Pfeiffer et al. 1999; Broer et al. 2000). A novel behaviour of system y+L in these cells is the finding that arginine is a better inhibitor of arginine flux than of lysine flux. Lysine on the other hand appears to have the same effect on both fluxes. This may be explained if there are different transport activities for each amino acid.


We are grateful to the Wellcome Trust for financial support.

C9

Lithium stimulates glucose transport in rat skeletal muscle cells by a mechanism that is independent of glycogen synthase kinase-3: the potential role of p38 MAP kinase

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Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that has been implicated in the regulation of diverse physiological responses, including glycogen metabolism (Frame & Cohen, 2001). GSK3 is inhibited by insulin and lithium and, like the hormone, Li has been shown to stimulate glucose transport and induce activation of glycogen synthase (GS) (Chen et al. 1998; Orena et al. 2000), leading to the suggestion that GSK3 inactivation may be of importance for the hormonal stimulation of glucose transport. In the present study we have used Li and SB-415286, a maleimide derivative that selectively inhibits GSK3 (Coghlan et al. 2000), to test this proposition in rat L6 myotubes.

Insulin stimulated glucose transport by ~2-fold and induced inactivation of GSK3 by ~50%. This inhibition in GSK3 led to an increase in GS activity by ~4.2-fold. Like insulin, Li and SB-415286 induced a significant reduction in GSK3 activity (by 73% and 97%, respectively) and caused a corresponding stimulation in GS, which was comparable to or greater than that elicited by insulin. L6 cells exposed to Li for 1 h induced a dose-dependent increase in glucose transport which was stimulated maximally to over 2-fold by 50 mM Li. Replacing Li with an equivalent concentration of sucrose did not mimic this effect suggesting that the response was not due to osmotic shock. In contrast, SB-415286 had no detectable effect on glucose transport. The Li-induced increase in glucose uptake was not sensitive to inhibitors of phosphoinositide 3-kinase, the classical mitogen-activated protein (MAP) kinase pathway or the mammalian target of rapamycin, but was suppressed completely by SB-203580, a p38 MAP kinase inhibitor. Li also induced a dose-dependent activation of p38 MAP kinase in L6 cells in a SB-203580 sensitive manner.

Our findings support a role for GSK3 in the chemical inactivation of GS which is sufficient to elicit a decrease in GS activity. We propose, based on the differential effects of Li and SB-415286, that GSK3 is unlikely to contribute towards the hormonal regulation of glucose transport in insulin target
tissues, as previously suggested (Orena et al. 2000; Chen et al. 1998). Instead, our data indicate that this stimulation arises through activation of p38 MAP kinase.


This work was supported by the MRC, BBSRC, Diabetes UK and GlaxoSmithKline.

C10
Diethylpyrocarbonate abolishes pH sensitivity of System A (SAT2) amino acid transporter
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The Na+-coupled System A amino acid transporters (SAT1–3) exhibit marked pH sensitivity, with influx depressed as external pH is lowered within the physiological range (Yao et al. 2000; Chaudhry et al. 2002); this may have important physiological implications for tissue amino acid and protein metabolism. (Bevington et al. 2002). We have investigated the possibility that this pH sensitivity relates to functionality of histidine residues within the transporter (SAT2) structure.

Figure 1. Effects of DEPC (2 mM, 10 min) on serine uptake in SAT2-expressing Xenopus oocytes. Values estimated from measurements at 6 different [NaCl] each using 8–11 oocytes.

Rat SAT2 (Yao et al. 2000) was studied by overexpression in Xenopus laevis oocytes. SAT2 cRNA (50 ng) was injected into oocytes and experiments were performed 2–3 days later. L-[3H]Serine (0.5 mM) influx through SAT2 was measured at pH 8 and 7 with NaCl concentrations of 0–100 mM (TMACl added to maintain osmolarity). Values shown are means ± S.E.M.

Pre-treatment of SAT2-expressing oocytes with the histidine-modifying reagent diethylpyrocarbonate (DEPC) resulted in substantial reduction of serine transport activity (2 mM DEPC for 10 min produced maximal effect of 71 ± 5% inhibition at pH 8; n = 8 expts) and loss of pH sensitivity over the pH range studied (Fig 1, right panel). DEPC treatment also blocks an increase in K₅₅ for Na⁺ activation of 0.5 mM serine transport seen on switching from pH 8 to 7 (Fig 1, left panel). Na⁺ was not required for DEPC to inhibit transporter activity but the SAT2 substrate serine (5 mM) offered partial (~50%) protection from the inactivating effects of DEPC. DEPC treatment abolishes pH sensitivity in the K₀₅ for Na⁺ activation of SAT2 without significantly affecting the value at pH 8. This is consistent with DEPC acting to alleviate an inhibitory allosteric effect of H⁺ on Na⁺ binding (Albers et al. 2001), rather than reduce competition between Na⁺ and H⁺ for a common binding site (Chaudhry et al. 2002). DEPC pre-treatment appears to reduce the rate of substrate translocation through SAT2, although the inability of DEPC to completely block transport suggests that its site of action is not directly at an essential substrate binding site. We suggest that DEPC binds to histidine residue(s) in the SAT2 protein at (or near) a putative H⁺ modifier site which may be in close proximity to the amino acid binding site (noting protection by serine).


We thank Dr J.D. Erickson (Louisiana State University) for rat SAT2 cDNA. This work was funded by MRC.

C17
Acute and chronic effects of hydrocortisone on Na⁺/H⁺ exchanger activity in human renal proximal tubule cells
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Glucocorticoids are reported to have stimulatory effects on renal proximal tubule Na⁺/H⁺ exchanger activity and on the expression of the NHE3 isoform. Here we have examined using spectrofluorescence microscopy, the effects of both acute and chronic exposure to hydrocortisone on luminal Na⁺/H⁺ exchanger activity in monolayers of primary cultured human proximal tubule (HPT) cells loaded with the pH-sensitive dye BCECF. The cells were acid-loaded using the NH₄Cl pulse method under sodium-free conditions and Na⁺/H⁺ exchanger activity was measured as the initial slope of pHi recovery (ΔpHi units per min, means ± S.E.M., Student’s paired t test) following the introduction of Na⁺ to the luminal bath. Chronic exposure of HPT cells to hydrocortisone (10 nM for 90 min) increased the rate of Na⁺-dependent pHi recovery twofold when compared to untreated controls (control ΔpHi = 0.10 ± 0.02, chronic hydrocortisone ΔpHi = 0.23 ± 0.03, n = 6, P < 0.001). Acute (seconds) exposure to hydrocortisone produced an inhibition of NHE activity. Under these conditions, when hydrocortisone was introduced into the luminal bath with sodium, the pHi recovery rate was reduced to 13% of control (control ΔpHi = 0.11 ± 0.02, acute hydrocortisone ΔpHi = 0.013 ± 0.012, n = 6, P < 0.005). Pre-incubation of HPT cells for 10 min with the PKC inhibitor chelerythrine chloride (1 µM) had no effect on the stimulation of NHE after chronic exposure to hydrocortisone but did block the acute inhibition of NHE. On the contrary, preincubation of the cells for 20 min with the PKA inhibitor (RP)-cAMP (200 µM) inhibited the chronic stimulation of NHE but had no effect on the acute inhibition of NHE.

We conclude that chronic activation of NHE by hydrocortisone occurs via a PKA-dependent signalling pathway whereas acute inhibition of NHE is via PKC.

This work was funded by the Health Research Board of Ireland.

All procedures accord with current National and local guidelines and the Declaration of Helsinki.
Potential stimulation and inhibition of the human CFTR Cl⁻ channel by the fluorescein derivative Bengal Rose B

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The fluorescein derivative phloxyzine B (4,5,6,7-tetrachloro-2',4',5',7'-tetrabromofluorescein) is a potent modulator of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel. Low micromolar concentrations of phloxyzine B stimulate CFTR Cl⁻ currents, whereas higher concentrations of the drug inhibit CFTR (Bachmann et al. 2000; Cai & Sheppard, 2002). To understand better the structure–activity relationship of fluorescein derivatives, we studied Bengal Rose B (4,5,6,7-tetrachloro-2',4',5',7'-tetrabromofluorescein), an agent with a chemical structure closely related to that of phloxyzine B.

The patch-clamp technique was used to investigate CFTR Cl⁻ channels in excised inside-out membrane patches from C127 cells stably expressing wild-type human CFTR (Cai & Sheppard, 2002). The pipette (external) solution contained 10 mM Cl⁻, whereas the bath (internal) solution contained 147 mM Cl⁻, PKA (75 mM) and ATP (0.3 mM) at 37°C; voltage was –50 mV. Following the activation of CFTR Cl⁻ currents by CAMP-dependent phosphorylation, drugs were added to the intracellular solution. Nanomolar concentrations of Bengal Rose B (0.1–1 μM) stimulated CFTR Cl⁻ currents, whereas low micromolar concentrations of the drug (2–10 μM) inhibited CFTR. Bengal Rose B (0.1 μM) increased open probability (Pₒ) from 0.31 ± 0.06 to 0.40 ± 0.08 (means ± S.E.M., n = 5, P < 0.05, Student’s paired t test), but caused a small, but significant decrease in current amplitude (i; P < 0.05). To determine how Bengal Rose B increased Pₒ, we investigated the drug’s effects on gating kinetics using the QuB software suite (Cai & Sheppard, 2002). Bengal Rose B (0.1 μM) increased Pₒ by greatly prolonging mean burst duration (MBD) (control MBD = 116 ± 8 ms; Bengal Rose B MBD = 232 ± 30 ms; n = 4; P < 0.05) without significantly altering the inter-burst interval (IBI) (control IBI = 219 ± 19 ms; Bengal Rose B IBI = 307 ± 37 ms; n = 4; P > 0.05). However, the characteristics of channel block by Bengal Rose B differed from those of phloxyzine B. Inhibition of CFTR by phloxyzine is time independent. In contrast, during constant exposure to the drug, Bengal Rose B (2–10 μM) caused a progressive decrease of i until channel activity completely disappeared (n = 10).

We interpret these data to suggest that (i) Bengal Rose B interacts directly with CFTR at multiple sites to modulate channel activity; (ii) Bengal Rose B might stimulate CFTR by a similar mechanism to that of phloxyzine B; (iii) by modifying the residues at positions 2',4',5',7' of fluorescein, potent modulators of CFTR might be developed.


This work was supported by the CF Trust and NKRF.

Voltage-dependent and -independent effects of intracellular pH on the human CFTR Cl⁻ channel

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl⁻ channel with complex regulation. We previously demonstrated that intracellular pH has multiple effects on the activity of CFTR (Chen et al. 2002). To elucidate how pH modulates the activity of CFTR, we investigated the effects of voltage on the CFTR Cl⁻ channel at different values of intracellular pH using excited inside-out membrane patches.
Membrane patches were bathed in symmetrical 147 mM Cl– solutions and the bath (internal) solution contained PKA (75 mM) and ATP (1 mM) at 37°C. To adjust the bath solution to pH 8.3 and pH 6.3, we used Tris and H2SO4, respectively. Data are means ± S.E.M. of n observations and statistical analyses were performed using Student’s paired t test.

In conclusion, our data demonstrate that pH-dependent changes in a metastatic cell line of human breast cancer (MCF-10A, MCF-7 and MDA-MB-231 cells cultured in RPMI 1640 medium with 10% fetal bovine serum). Voltage-gated currents were activated by applying depolarizing pulses in steps of 10 mV from a holding potential of −100 mV. The basic electrophysiological observations are summarised in Table 1. In essence, an inward current was present in 29% (n = 56) of the strongly tetrodotoxin (TTX) sensitive, Nav1.7 being the predominant subtype expressed (Diss et al. 2001). Furthermore, blocking VGSC activity in these cells suppressed a variety of cellular behaviours involved in the metastatic cascade, such as basic and galvanotactic motility (Fraser et al. 1998; Djamgoz et al. 2001), endocytic membrane activity (Mycielska et al. 1997). In the present study, we have investigated whether a similar situation may exist in breast cancer (BCa) cell lines of markedly different metastatic ability.

Whole-cell patch clamp recordings were obtained from MCF-10A, MCF-7 and MDA-MB-231 cells cultured in RPMI 1640 medium with 10% fetal bovine serum. Voltage-gated currents were activated by applying depolarizing pulses in steps of 10 mV from a holding potential of −100 mV. The basic electrophysiological observations are summarised in Table 1. In essence, an inward current was present in 29% (n = 56) of the strongly
metastatic MDA-MB-231 cells, but in none of the other cell lines tested. On the other hand, outward current density was inversely related to the cells’ metastatic ability, such that only very small outward currents were recorded in the MDA-MB-231 cells. The inward currents were blocked by TTX in a dose-dependent fashion, the IC50 being greater than 1 μM, i.e. the VGSC was TTX-resistant. Indeed, semi-quantitative RT-PCR analyses revealed that Nav1.5 was the predominant VGSC expressed in this cell line. The possible role of VGSC activity in metastasis was tested in Matrigel invasion experiments (Boyden chambers with chemo-attractant). These functional assays showed that pre-treating cells with 10 μM TTX reduced their invasiveness by some 40%.

| Table 1. Voltage-gated channel expression in human breast cancer cell lines of varying metastatic ability |
|-----------------|-----------------|-----------------|-----------------|
| Cell type       | Metastatic potential | Voltage-activated inward (Na+) current | Voltage-activated outward current |
| MCF-10A         | None             | None             | Very large       |
| MCF-7           | None/weak        | None             | Medium           |
| MDA-MB-231      | Strong           | Yes              | Very small       |

In conclusion: (1) Strongly metastatic BCa cells are potentially electrically excitable and (2) VGSC activity could accelerate metastasis in BCa, as found previously for PCa cells.

Fraser SP et al. (1998). J Physiol 513P, 131P.

This work was supported by Cancer Research UK and the Pro Cancer Research Fund.

C23

Open-channel block of the human CFTR Cl– channel by the loop diuretic furosemide

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The loop diuretic furosemide is widely used to inhibit the Na+-K+-2Cl– cotransporter (Haas & Forbush, 2000). However, Venglarik (1997) demonstrated that furosemide inhibits the cystic fibrosis transmembrane conductance regulator (CFTR) Cl– channel. To investigate the mechanism of furosemide inhibition of the CFTR Cl– channel, we used inside-out membrane patches excised from C127 cells stably expressing wild-type human CFTR (for Methods, see Lansdell et al. 2000). The pipette (external) solution contained 10 mM Cl– and the bath (internal) solution contained 147 mM Cl–, 0.3 or 1 mM ATP and 75 mM PKA at 37°C; voltage was –50 mV. We expressed data as means ± S.E.M. of n observations and we performed statistical analyses using Student’s unpaired t test.

When added to the internal solution, furosemide (100 μM) caused a flickery block of single CFTR Cl– channels that decreased both open probability (Po; control, 0.41 ± 0.03; furosemide (100 μM), 0.17 ± 0.05; n = 6), and single-channel current amplitude (i; control, –0.76 ± 0.01 pA; furosemide (100 μM), –0.63 ± 0.03 pA; n = 6). Block was readily reversible on washing (n = 6). To learn whether furosemide is an open-channel blocker of CFTRs, we examined the voltage dependence of furosemide inhibition. When excised patches were bathed in symmetrical 147 mM Cl– solutions, furosemide (100 μM) strongly inhibited CFTR Cl– currents at negative voltages. However, channel block was completely relieved at positive voltages. Using current values in the absence and presence of furosemide, the voltage-dependent dissociation constant (Kd) for furosemide inhibition at 0 mV was calculated to be 332 ± 29 μM (n = 5). This voltage dependence of inhibition suggests that furosemide binds within the electric field of the membrane possibly within the channel pore. If the binding site is located within the channel pore, the passage of Cl– ions through the channel pore would be predicted to interfere with furosemide inhibition. Consistent with this idea, when the external [Cl–] was reduced to 10 mM, the Kd of furosemide inhibition at 0 mV decreased to 156 ± 15 μM (n = 6; P < 0.01).

These results suggest that furosemide is an open-channel blocker of the CFTR Cl– channel. They also suggest that furosemide and Cl– ions might compete for a common binding site.


This work was supported by the CF Trust and NKRF.

PC1

Regulatory volume decrease by in situ human chondrocytes

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Changes to chondrocyte volume have a deleterious effect on the metabolism of the extracellular matrix of articular cartilage, possibly leading to the cartilage loss characteristic of osteoarthritis (OA; Urban et al. 1993). Using confocal laser scanning microscopy (CLSM) we have reported that chondrocytes from areas of degenerate tissue are larger than those from non-degenerate regions (Bush & Hall, 2003). It is possible that this cell swelling results from the inability of the cells to undergo regulatory volume decrease (RVD). Here, we have tested the RVD capacity of in situ human chondrocytes within non-degenerate and degenerate cartilage.

Cartilage from human tibial plateau (with Ethical permission) was obtained at total knee replacement surgery and cultured in Dulbecco’s modified Eagle’s medium (280 mosmol l–1). The fluorescent dye calcine was incorporated into chondrocytes (calcine-AM; 5 μM, 37°C, 30 min). The resting volume of mid zone (MZ) chondrocytes, and their RVD capacity during a rapid (< 30 s) osmotic challenge (280 to 180 mosmol l–1; 21°C) were measured from CLSM images (Zeiss LSM 510, Axioskop, X 63 ceramic water dipping lens) using analysis software (Bitplane, Zurich). Chondrocyte volumes were reported as the percentage change compared to the value before osmotic challenge and plotted against time. Data are means ± S.E.M. (total number of joints [total number of cells]).

Paired samples of relatively non-degenerate articular cartilage (ACND, (3[21])) or degenerate articular cartilage (ACD, (3[20])); demonstrating surface fibrillation) were taken from three joints. The resting volumes of cells before hypo-osmotic challenge were larger in ACND, 792 ± 44 μm3 compared to 671 ± 41 μm3 for ACD (P < 0.002; Students’ paired t test). When exposed to a hypo-osmotic challenge, there was no difference in the extent of cell swelling,
28 ± 4 % and 36 ± 3 % for ACND and ACID, respectively (P < 0.05). After 20 min post-osmotic challenge, cell volume had recovered from the point of maximal swelling by 32 ± 12 % and 30 ± 17 % for ACND and ACID, respectively (P < 0.05).

Human articular chondrocytes within degenerate cartilage (ACID) are larger than those in non-degenerate tissue (ACND), but there was no difference in the response to the hypotonic challenge. The extent of cell swelling and the rate of volume recovery by RVD were similar. These data show that the RVD mechanism activated in response to an acute hypotonic challenge is not compromised in chondrocytes within degenerate cartilage.

Urban JPG et al. (1993), J Cell Physiol 154, 262–270.

This work was supported by the Arthritis Research Campaign (H0621).
We thank Mr J. Aderinto for helping with the cartilage samples.

All procedures accord with current local guidelines and the Declaration of Helsinki.

PC2

Acid secretion by surface epithelium of isolated porcine distal airways

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Secretion of HCO₃⁻ by airway submucosal glands is essential for normal liquid and mucus secretion (Inglis et al. 1998). Since the liquid bathing the airway surface is fairly acidic, we have proposed that the surface epithelium may acidify HCO₃⁻-rich glandular fluid. We previously showed that isolated distal bronchi, containing both glandular and surface epithelium, can both acidify and alkalise their lumen (Inglis et al. 2003). The aim of the current study was to investigate whether the acidification arises from ion transport in the surface or glandular epithelium.

Porcine distal bronchi were isolated from pigs humanely killed with an overdose of sodium pentobarbital, cannulated in a bath containing HCO₃⁻-buffered solution and perfused (3ml min⁻¹) with similar solution, in which NaCl replaced NaHCO₃⁻. This solution was lightly buffered (buffering capacity 0.6 mM pH unit⁻¹) with KH₂PO₄ and NaOH to pH ~7, gassed with 100 % O₂ to eliminate dissolved CO₂ and stirred vigorously. The pH of this circulating luminal solution (10 ml) was monitored continuously. Transepithelial PD was measured using a luminal microelectrode referenced to the bathing solution, connected to an electrometer.

As previously shown (Inglis et al. 2003), upon perfusion through the airway luminal pH initially fell by 0.057 ± 0.009 pH units ([H⁺] increase 2.54 ± 0.45 μmol l⁻¹ (means ± s.e.m., n = 7)) before stabilising. Since removing the surface epithelium removes the primary resistive barrier to passive HCO₃⁻ transport into the lumen, subsequent experiments were carried out in the absence of HCO₃⁻. HCO₃⁻ removal significantly reduced the acidification (pH fell by 0.035 ± 0.007 pH units; [H⁺] increase 0.782 ± 0.1 μmol l⁻¹ (means ± s.e.m., n = 7, P < 0.05, Student’s paired t test used throughout). The acidification was further inhibited (65.0 ± 3.8 %, n = 5, P < 0.05) by luminal bafilomycin A₁ (100 nm), a vH⁺-ATPase blocker. Previous results showed that bafilomycin A₁ had similar effects in the presence of HCO₃⁻ (Inglis et al. 2003). To remove surface epithelium, a nylon brush was pushed through the airway lumen. Histological analyses revealed this successfully removed surface epithelium and left underlying glandular epithelium relatively intact (see Fig. 1). Such treatment abolished transepithelial PD (control bronchi -6.3 ± 1.7 mV; epithelium-striped bronchi 0.1 ± 0.4 mV, n = 5, P < 0.05) and significantly reduced the acidification (control bronchi 0.022 ± 0.003 pH units; epithelium-striped bronchi 0.009 ± 0.006 pH units, n = 5, P < 0.05).

PC3

Expression of KCNE1 and KCNQ1 mRNA in the proximal tubule of mouse kidney

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In several tissues, the proteins KCNQ1 and KCNQ1 (Lk) combine to form voltage-gated K⁺ channels. In the proximal tubule (PT) of murine kidney, KCNQ1 mediates a cell to lumen K⁺ flux which may serve to counteract membrane depolarization due to electrogenic Na⁺-coupled uptake of glucose or amino acids (Vallon et al. 2001). The aim of the present study was to demonstrate expression of KCN1 and KCNQ1 mRNA in...
isolated mouse PTs and also to determine KCNE1 protein expression in the kidney.

129/SV mice were killed by cervical dislocation and the kidneys rapidly removed for extraction of total RNA or for protein extraction. RT-PCR was performed on RNA extracted from total mouse kidney (TMK: n = 5 preparations) and from S1 and S2 segments of proximal tubules (n = 5 preparations) isolated by enzymatic digestion of cortical slices. For PCR, we used oligonucleotide primers designed against murine KCNE1 and KCNQ1 (Grahammer et al. 2001). The predicted size of PCR products was 355 and 421 base pairs (bp), respectively. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide fluorescence (302 nm).

Western blotting was performed on lysates (n = 3) extracted from TMK centrifuged at 15 000 g representing a crude membrane fraction, and then centrifuged at 50 000 g to obtain a cytosolic fraction. Extracted proteins were separated by 4–12% gradient PAGE, and then electroblotted on to a nitrocellulose membrane. The membrane was incubated with an antibody against KCNE1 developed ‘in house’ against the C-terminal peptide of KCNE1 and visualized by standard chemiluminescent methods.

PCR products of 355 and 421 bp corresponding to KCNE1 and KCNQ1, respectively, were obtained from PT. Western blotting for KCNE1 revealed a major band at 15 kDa predominantly in the membrane fraction of the mouse kidney samples. These experiments indicate that mRNAs for KCNQ1 along with KCNE1 are expressed in mouse proximal tubules and the protein for KCNE1 is also localized in mouse kidney membrane fractions. These results are consistent with the hypothesis that KCNQ1 and KCNE1 have a role in setting the membrane potential in the proximal tubule.


The Wellcome Trust financed this work.

All procedures accord with current UK legislation.

PC4
Sub-cellular localisation of the Cl− channel mediator, mCLCA1: correlation with the intracellular Cl− channel mCLC-5
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Mouse renal inner medullary collecting duct cells (mIMCD-3 cell-line) express several members of the CLCA protein family (including mCLCA1) that mediate Cl− channel activity (Stewart et al. 2001). The voltage-dependent Cl− channel mCLC-5 is also expressed in mIMCD-3 cells within acidic endosomes (Sayer et al. 2001). Here we report subcellular localisation of CLCA1 and correlate this to that of mCLC-5.

mIMCD-3 cells were transiently transfected alone or in combination with expression vectors for GFP fusion proteins for mCLCA1 (Sayer et al. 2000) and mCLC-5 (Sayer et al. 2001) (pCDNA3.1/mCLCA1-CT-RFP, or pDsRed1/mCLC-5CT, Clontech), using Lipofectamine 2000 (Life Technologies). Positive transfectants were identified using a Leica confocal laser imaging microscope equipped (CLSM) with a Kr–Ar laser by their green/red fluorescence and analysed 24–48 h post-transfection. In order to identify plasma membrane in mCLCA1-GFP transfectants, cells were preincubated for 2 min at room temperature with TRITC-wheat germ agglutinin (WGA) in phosphate-buffered saline (PBS) or complete medium (Ham’s F12/DMEM) at 50 mg ml−1. Alternatively, in order to identify acidic endosomes, cells were incubated with 75 nM lysotracker red (Molecular Probes) for 30 min in PBS or complete medium. Optical sections of positive transfectants were collected to allow analysis of co-localisation of mCLCA1-GFP fluorescence with plasma membrane, acidic endosomes and mCLC-5.

mCLCA1-GFP fluorescence at 24/48 h post-transfection was primarily associated with intracellular vesicular structures. A minor proportion of CLCA1-GFP fluorescence was co-localised with TRITC-WGA, confirming the presence of mCLCA1-GFP at the plasma membrane. mCLCA1-GFP fluorescence showed separation from both acidic endosomes and mCLC5-GFP, with only a minor overlap. The presence of mCLCA1 at the plasma membrane is consistent with mCLCA1 conferring Cl− channel activity or acting as a Cl− channel regulator. The virtual separation of mCLC-5 from mCLCA1 suggests that CLCA proteins could not compensate for loss of CLC-5 function in IMCD cells.


This work was supported by the NCKRF and the NKRF

PC5
Zinc stimulates the promoter activity of the divalent metal transporter (DMT1) gene in human intestinal Caco-2 cells
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We have previously demonstrated that exposure of the human intestinal Caco-2 cell line to zinc stimulates iron uptake via an increase in DMT1 transporter expression (Yamaji et al. 2001). The cellular mechanisms involved in the up regulation of DMT1 by zinc are still unclear, though one possibility is that activation occurs via interaction with putative metal response elements (MRE) residing in the 5′ promoter region of the DMT1 gene (Lee et al. 1998). To test this possibility, we have investigated the effect of zinc on the activity of the DMT1 promoter using a reporter gene assay.

Caco-2 cells were seeded at a density of 6 × 103 cells cm−2 into 24-well plates. After 3 days cells were transfected using the CaPO4 method with pGL3 plasmid (Promega, UK) that contained 1.6 kb of the DMT1 promoter cloned in front of a luciferase reporter gene. Three days following transfection, cells were exposed to zinc (100 μM) for 24 h and luciferase activity in cell lysates was measured by luminescence.

Zinc stimulation of Caco-2 cells transfected with the DMT1 promoter construct significantly increased luciferase activity compared with unstimulated cells (control 928 ± 79 a.u. vs. +Zn 4702 ± 348 a.u., means ± S.E.M., n = 6, P < 0.0001, Student’s unpaired t test). This suggests that zinc may promote the absorption of iron by Caco-2 cell monolayers by activating a transcription factor that can interact with a specific consensus
sequence within the DMT1 promoter. The nature of the transcription factor is unknown but the zinc-inducible MTF-1 that binds to MRE sequences in several genes (Andrews, 2001) is a possible candidate. Further studies are underway in our laboratory to further identify the mechanisms involved in this response.

This work was funded by BBSRC grant (90/D13400).

PC6

DMT1 protein expression in the apical membrane of human intestinal Caco-2 cells is rapidly decreased following exposure to iron
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The transporter DMT1 mediates uptake of iron from the diet by intestinal enterocytes. Recently, we have shown that DMT1 protein expression in the plasma membrane of human intestinal Caco-2 cells is decreased by exposure to iron in a dose-dependent fashion (Sharp et al. 2002). Intriguingly, whole cell levels of DMT1 do not change, suggesting that the transporter may be internalised into an intracellular compartment. In this study we have investigated the time course of the iron-dependent decrease in DMT1 in Caco-2 cells to determine whether changes in expression might occur within a physiologically relevant period coincident with the digestion and processing of a meal in the normal gastrointestinal tract.
Caco-2 cells were grown in 25 cm² flasks for 21 days. Cells were incubated with 100 μM FeCl₃ for up to 24 h. At the end of the experimental period, whole cell and plasma membrane proteins were isolated and utilised for Western blotting and total RNA extracted and subjected to RT-PCR for DMT1. In some experiments, membrane proteins were biotinylated prior to exposure to iron. In these studies, at the end of the incubation period, cells were lysed and the amount of biotinylated DMT1 in the cytosol determined following immunoprecipitation with DMT1 antibody, protein separation by western blotting and visualisation with streptavidin–HRP and enhanced chemiluminescence.

Plasma membrane DMT1 was significantly reduced by 4 h exposure to iron (0 h, 91.4 ± 6.7 a.u.; 4 h, 52.9 ± 11.1 a.u., means ± s.e.m., n = 4, P = 0.025, Student’s unpaired t test). However, whole cell levels of DMT1 were unaltered by iron exposure. DMT1 mRNA levels isolated from control and iron-treated cells were not significantly different at this time. Interestingly, there was an increase in biotinylated DMT1 in the cytosol following exposure to iron (control 8.3 μM, +Fe 23.7 μM, n = 2). Taken together, these data suggest that the initial cellular response to elevated iron involves a decrease in apical membrane expression of DMT1, perhaps due to internalisation of the transporter into an intracellular compartment. These changes are rapid (between 1 and 4 h following exposure to iron) and could occur within the time scale for the digestion and processing of a meal.

This work was funded by BBSRC (project grant 90/D13400).

PC7

D-Glucose does not inhibit adenosine transport in fibroblasts from Cat2- or iNOS-deficient mice
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Expression of equilibrative nucleoside transporters 1 (ENT1, inhibited by nanomolar nitrobenzylthioninosine, NBMPR) is reduced by d-glucose, an effect associated with activation of the L-arginine/NO pathway (Parodi et al. 2002). We investigated whether the inhibitory effect of elevated d-glucose on adenosine transport depends on expression of cationic amino acid transporters 2 (CAT2) and the inducible NO synthase (iNOS).

Mouse embryonic fibroblasts (MEFs) from humanely killed wild-type, Cat2- and iNOS-deficient mouse (Ethics Committee approval was obtained) were cultured (24 h) in medium 199 (M199), containing 10% newborn calf serum, 3.2 mM L-glutamine, and 5 or 25 mM d-glucose. Adenosine transport ([2,8,5-3H]adenosine, 60 Ci mmol⁻¹, 0–500 μM, 2 μCi ml⁻¹, 22°C, 20 s) was measured in cells incubated (0–24 h) with M199 in the absence or presence of cytokines (10 ng ml⁻¹ IL-1β + 10 ng ml⁻¹ TNF-α + 20 I.U. ml⁻¹ INF-γ), N²-nitro-L-arginine methyl ester (L-NAME, 100 μM), S-nitroso-N-acetyl-L-d-penicillamine (SNAP, 10 μM), or NBMPR (1–1000 μM). CAT2 and iNOS mRNA was amplified by reverse transcriptase-polymerase chain reactions. iNOS activity was determined by L-[3H]arginine methyl ester (L-NAME, 4 μCi ml⁻¹, 30 min) (Casanello & Sobrevia, 2002).

Adenosine transport in wild-type, Cat2-/- and iNOS-/- MEFs (1.2 ± 0.12, 1.1 ± 0.06 and 1.2 ± 0.2 pmol (mg protein)⁻¹ min⁻¹, respectively; means ± s.e.m., n = 8–16) was inhibited (P < 0.05, Student’s unpaired t test) by 1 mM NBMPR (0.1 ± 0.02, 0.2 ± 0.01 and 0.2 ± 0.02 pmol (mg protein)⁻¹ min⁻¹, respectively). Elevated d-glucose reduced the Vₘₕ (12 ± 2 vs 43 ± 5 pmol (mg protein)⁻¹ min⁻¹, P < 0.05, n = 12) with non-significant changes in the apparent Kₘ (84 ± 12 vs 101 ± 24 μM) for NBMPR-sensitive adenosine transport in wild-type MEFs. Adenosine transport in Cat2-/- or iNOS-/- MEFs was unaltered (P > 0.05). Cytokine-activated wild-type MEFs cultured in 5 mM d-glucose reduced adenosine transport to similar values determined in 25 mM d-glucose. ENT1 mRNA level was reduced (47 ± 4%) by 25 mM d-glucose only in wild-type MEFs. D-Glucose or cytokine effects on adenosine transport activity and expression were blocked by L-NAME, and mimicked by SNAP. These results suggest that adenosine transport via ENT1 depends on expression of CAT2 and iNOS in MEFs.


This work was supported by FONDECYT (1030781, 1030607, 1000354, 7000354) and DIUC-University of Concepción (201.084.003-L)–Chile, The Welcome Trust (UK) and Fulbright Commission (USA).

All procedures accord with current local guidelines.

This work was funded by BBSRC (project grant 90/D13400).

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PC8
Effects of purine receptor agonists on whole cell currents from frog isolated proximal tubule cells

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Extracellular ATP activates P2X purinoceptors, a class of receptors that form Ca\(^{2+}\) permeable channels. A previous study has demonstrated that inhibitors of one of these, P2X\(_{4}\), block volume regulation in frog isolated proximal tubule cells (Davies & Robson, 2002). The aim of the following study was to identify whether whole cell ATP-activated currents observed in the frog proximal cells were mediated by P2X\(_{4}\).

Frogs were killed humanely by cervical dislocation and single proximal tubule cells were isolated from frog kidneys by enzymic digestion (Hunter, 1989). Standard patch clamp techniques were used to gain whole cell patches via the basolateral membrane. The pipette solution contained (mM): 100 NaCl, 2 MgCl\(_2\), 0.5 EGTA and 10 Hepes (NaOH). The bath solution contained (mM): 100 NaCl, 0.5 MgCl\(_2\), 0.5 CaCl\(_2\), and 100 Hepes (NaOH). Whole cell potential was held at \(-100\) mV and was then ramped between \(-100\) and \(+20\) mV. To examine agonist potency, patches were exposed to either 500 \(\mu\)M ATP and 500 \(\mu\)M 2-MeSATP suggest that the activated conductance may be mediated by either P2X\(_{4}\), P2X\(_{5}\) or P2X\(_{6}\). Further studies are needed to distinguish between these receptors.

Addition of 500 \(\mu\)M ATP to the extracellular surface of patches assumed at the 5% level.

These data suggest that single proximal tubule cells isolated from frog kidney contain an ATP-activated conductance. The relative potencies of the P2X agonists ATP and BzATP suggest that the conductance is not due to P2X\(_{4}\). Further studies are needed to distinguish between these receptors.


This work was supported by the Wellcome Trust.

All procedures accord with current UK legislation.

PC9
CAMP-dependent regulation of basolateral glycy1-sarcosine uptake by human intestinal Caco-2 cell monolayers

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The activity of the proton-coupled apical peptide transporter of human intestinal cells (hPepT1) is regulated indirectly through a functional couple with the apical Na\(^{+}/H^+\) exchanger NHE3 (Thwaites et al. 2002). In contrast, the peptide transporter present at the basolateral membrane shows a different dependence upon pH and is directly dependent upon medium Na\(^{+}\) but independent of basolateral NHE1 activity (Henderson et al. 2002). We now report that the basolateral peptide transporter is negatively regulated by vasoactive intestinal peptide (VIP) acting via intracellular cAMP.

To examine agonist potency, patches were exposed to either 500 \(\mu\)M ATP and 500 \(\mu\)M 2-MeSATP. Under both circumstances the order of exposure to the agonists was altered with each patch. Data are expressed as means ± S.E.M. Statistical analysis was performed using Student’s paired t test and significance was assumed at the 5% level.

Addition of 500 \(\mu\)M ATP to the extracellular surface of patches increased outward conductance (\(G_{\text{out}}\)) and inward conductance (\(G_{\text{in}}\)) by 9.55 ± 1.61 and 5.45 ± 1.00 \(\mu\)S cm\(^{-2}\), respectively (\(n = 20\)). In paired patches, ATP increased \(G_{\text{out}}\) while BzATP was without effect. The ATP-activated \(G_{\text{out}}\) was 7.11 ± 1.97 \(\mu\)S cm\(^{-2}\) (\(n = 12\)), while BzATP did not give a significant shift in \(G_{\text{out}}\) (0.14 ± 0.95 \(\mu\)S cm\(^{-2}\)). In contrast, both ATP and 2-MeSATP increased \(G_{\text{out}}\) by 11.1 ± 1.35 \(\mu\)S cm\(^{-2}\) and 3.64 ± 0.89 \(\mu\)S cm\(^{-2}\), respectively (\(n = 25\)). The ATP-activated conductance was significantly greater than the 2-MeSATP activated conductance.

These data suggest that single proximal tubule cells isolated from frog kidney contain an ATP-activated conductance. The relative potencies of the P2X agonists ATP and BzATP suggest that the conductance is not due to P2X\(_{4}\). Further studies are needed to distinguish between these receptors.


This work was supported by the Wellcome Trust.

All procedures accord with current UK legislation.


F.D.H.’s studentship was sponsored by GlaxoSmithKline.
The antimicrobial peptide hepcidin decreases iron uptake by human intestinal Caco-2 cells

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Hepcidin is a 25 amino acid peptide produced in the liver whose expression is increased by iron loading and decreased in iron deficiency (Pigeon et al. 2001). This site of synthesis and the dramatic regulation by iron has led to the suggestion that hepcidin might be the master controller of iron metabolism, re-locating information about the status of the body iron stores to the intestine and regulating absorption accordingly. The mode of action of hepcidin is still unclear. In this study we have utilised the Caco-2 cell model of human intestinal epithelial cells to investigate the possibility that hepcidin might interact directly with the epithelium.

Cells were cultured in Transwell plates for 21 days. For the final 24 h of the culture period, human synthetic hepcidin (30 µg ml⁻¹) was added to the basolateral medium. At the end of the incubation period cells were either used to measure ⁵⁵Fe transport across the Caco-2 cell monolayers, processed for Western blotting for the iron transport proteins DMT1 and IREG1, or used as a source of RNA to determine changes in transporter expression by Real-Time RT-PCR.

Following exposure to hepcidin, iron uptake across the apical membrane of Caco-2 cells was significantly decreased (control 453.3 ± 47.0 pmol cm⁻² h⁻¹; +hepcidin 268.3 ± 63.8 pmol cm⁻² h⁻¹, means ± S.E.M. n = 6, P = 0.04 Student’s unpaired t test). Efflux across the basolateral membrane was unaffected by hepcidin treatment. In agreement with the transport data, the expression of the apical membrane transporter DMT1 was decreased by hepcidin treatment at both the protein and mRNA level, whereas expression of IREG1, the basolateral efflux protein, was unaffected.


PC10

Intracellular pH regulation in isolated human colonic crypts

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In the human colon the removal of HCO₃⁻ from the bathing medium reduces the cAMP-dependent secretory response by 60% (Taylor et al. 2001). As this HCO₃⁻ dependent secretion is independent of Cl⁻ and inhibited by serosal 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) it is likely that it is mediated via a basolateral NaHCO₃ cotransporter. Therefore, we have used measurements of intracellular pH (pHi) to determine if a NaHCO₃ cotransporter is present in the secretory cells of the human colon.

All measurements used crypts isolated from colonoscopy biopsies collected from the descending and sigmoid colon. Isolated crypts were loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF) and pH, was measured in the basi-third of the crypts at 37°C. To characterize the pH, regulatory mechanisms, cells were acidified with an NH₄Cl pre-pulse and the activity of pH, regulatory mechanism quantified by the initial rate of recovery of pHi, expressed as ApH units min⁻¹. All data are given as means ± S.E.M. and n = number of crypts. The experiments were approved by the Otago Ethics Committee, Dunedin, New Zealand.

In HCO₃⁻-free Ringer solution, pHi recovery was dependent upon the presence of Na⁺ (ApH units min⁻¹ in presence = 0.19 ± 0.02, n = 12 and absence of Na⁺ = 0.022 ± 0.007, n = 15; P < 0.05, Student’s unpaired t test). It was also inhibited in a dose-dependent manner by amiloride (Kᵢ = 8 µM) with complete inhibition occurring at 0.5 mM (ApH units min⁻¹ = 0.004 ± 0.002, n = 4). In the presence of HCO₃⁻/CO₂ recovery of pHi, was also dependent upon Na⁺ (ApH units min⁻¹ in presence = 0.100 ± 0.013, n = 6 and absence of Na⁺ = −0.023 ± 0.006, n = 11; P < 0.01, ANOVA, Dunnett’s post test). However, although the recovery was inhibited by 0.5 mM amiloride (ApH units min⁻¹ 0.0215 ± 0.005, n = 6, P < 0.01, ANOVA, Dunnett’s post test), there was evidence of amiloride-insensitive pH, recovery. This amiloride-insensitive pHi, recovery was inhibited by DIDS. Following exposure to NH₄Cl and Na⁺-free Ringer solution the rate of recovery of pHi, in the presence of 0.5 mM amiloride was 0.0789 ± 0.009 pH units min⁻¹ (n = 18), whereas in the presence of amiloride plus 250 µM DIDS this was reduced to 0.0176 ± 0.005 pH units min⁻¹ (n = 9, P < 0.01, unpaired Student’s t test).

These data demonstrate that the dominant mechanism for regulation of pH, in human colonic crypts under basal conditions is a Na⁺/H⁺ exchanger. There is also a small contribution from a HCO₃⁻-dependent transporter, most likely a NaHCO₃ cotransporter. It remains to be established whether this is stimulated by secretagogues and so contributes to the secretory response of the intact colon.


This work was funded by the University of Otago.

All procedures accord with current local guidelines and the Declaration of Helsinki.

PC12

Protein kinase C mediates the inhibitory effect of substance P on pancreatic ductal HCO₃⁻ secretion

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The regulatory pathways that stimulate pancreatic ductal HCO₃⁻ secretion are well described, but less is known about inhibitory pathways. Inhibitory pathways may be important in terms of limiting the hydrostatic pressure within the ducts (so preventing leakage of enzymes into the parenchyma of the gland), and in terms of switching off pancreatic secretion after a meal. Substance P (SP) inhibits secretin-stimulated HCO₃⁻ secretion (Ashton et al. 1990), and we have recently reported that SP exerts its effect by inhibiting an apical Cl⁻/HCO₃⁻ exchanger in the duct cell (Hegyi et al. 2001). The purpose of this study was to identify the intracellular signalling pathway utilized by SP.

Guinea-pigs (150–250 g) were humanely killed, the pancreas was removed and small intra/interlobular ducts were isolated and...
cultured overnight as previously described (Argent et al. 1986). The rate of HCO\textsubscript{3}\textsuperscript{-} secretion was determined by measuring the initial rate of intracellular acidification (using BCECF) following sudden block of basolateral NaHCO\textsubscript{3} cotransporters and Na\textsuperscript{+}/H\textsuperscript{+} exchangers with DIDS (100 \mu m) and amiloride (200 \mu m), respectively (Szalmay et al. 2001). The buffering capacity of duct cells was estimated and the rate of transmembrane H\textsuperscript{+} flux, J_H, was calculated. Since H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} will largely be derived from H_, HCO\textsubscript{3}. All the experiments were performed in HCO\textsubscript{3}.-buffered Ringer solution at 37°C.

Secretin (10 nM) elevated the basal rate of HCO\textsubscript{3}\textsuperscript{-} secretion from 2.18 ± 0.2 mM min\textsuperscript{-1} to 6.3 ± 1.5 mM min\textsuperscript{-1} (n = 6 ducts, P < 0.05, means ± S.E.M. and Student’s t-test). SP (20 nM) had no effect on the basal rate, but totally inhibited the secretin-stimulated elevation of J_H (n = 6 ducts). This inhibitory effect of SP could be relieved by 20 nM spantide, a SP receptor antagonist (n = 6 ducts). Phorbol 12,13-dibutyrate (100 nM), an activator of protein kinase C (PKC), reduced the basal rate of HCO\textsubscript{3}\textsuperscript{-} secretion by 38% (n = 7 ducts), and totally blocked secretin-stimulated J_H (n = 6 ducts).

In addition, bisindolylmaleimide (1 \mu m), an inhibitor of PKC, partially relieved the inhibitory effect of SP on secretin-stimulated J_H.

Our data suggest that SP exerts its inhibitory effect on the duct cell by activating PKC, which then inhibits apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange.


This work was funded by The Wellcome Trust (Grant No. 022618) and the Hungarian Scientific Research Fund (Grant No. D42188).

All procedures accord with current UK legislation.

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PC15
Calmodulin binding proteins and nuclear pores shape the calcium induced translocation of calmodulin in pancreatic acinar cells
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It has been shown in pancreatic acinar cells (Craske et al. 1999) that calmodulin (CaM) undergoes intracellular redistribution after application of the calcium-releasing agonists, acetylcholine (ACh) and cholecystokinin (CCK). A large cytosolic calcium transient generated by a super-maximal dose of ACh caused a rapid rise of [CaM] in apical region and a rapid drop in the basal region while the [CaM] in the nucleus rose more slowly, and lasted long after the termination of the calcium transient. Global cytosolic calcium spikes generated by 5 pm CCK caused [CaM] spikes in the apical region that resembled cytosolic calcium spikes, and [CaM] in the basal region showed the mirror image of the apical [CaM] spikes with smaller magnitude. On the other hand, [CaM] in the nucleus showed a delayed, slower and steadier increase that stabilized in about 100 s and its fluctuations were much smaller than those of [CaM] in apical or basal region.

In order to understand these behaviours of [CaM] during the agonist stimulations, we developed computational models using FEMLAB, an interactive environment for modelling mathematical problems based on a system of coupled partial differential equations. The model had one-dimensional geometry with three subdomains which represented the nucleus, apical and basal cytosolic regions. We assumed that calcium-free calmodulin (apoCaM) and calcium-bound calmodulin (CaCaM) could diffuse freely in the cytosol or in the nucleus, or bind to non-diffusible binding partners (apoCaM-binding proteins and CaCaM-binding proteins, respectively). It was also assumed that the diffusion between the cytosol and the nucleus was limited by nuclear pores whose permeability for CaM was dependent on cytosolic calcium concentration. Calcium concentration was set to be spatially uniform.

Our computational models showed (1) that a heterogeneous distribution of calmodulin binding proteins and the calcium dependence of the permeability of nuclear pores for CaM were the basis of the translocation of CaM induced by the change of cytosolic calcium, (2) a positive effect of calcium on the permeability for CaM explaining the delay in the accumulation of CaM in the nucleus which was observed experimentally, and (3) that binding proteins for apoCaM worked against the translocation by stabilizing free CaM concentration.

Our models can be used for the analysis of translocation of other calcium binding proteins with different properties (e.g. translocation from the cytosol to the plasma membrane or organellar membranes).


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PC16
Several charged residues in the extracellular domain of TASK-2 contribute to pH sensing
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TASK-2 is a member of the two-pore domain K\textsuperscript{+} channel (K\textsubscript{2p}) family and is sensitive to changes in extracellular pH; currents are maximal at alkaline pH but are progressively inhibited as the pH decreases (Reyes et al. 1998). TASK-2 is located principally in epithelial tissues, and is present in all nephron segments (Morton et al. 2002). TASK-2 has high sequence homology with TASK-4, but lower similarity to TASK-1 and -3. In TASK-1 and -3, mutation of a charged amino acid, histidine, at position 98 abolished the pH sensitivity of this channel (Kim et al. 2000; Morton et al. 2003). However, no such homologous residue is present in TASK-2 implying a different pH sensing mechanism. pH sensing must involve titration of charged residues, and thus we have examined the involvement of charged residues in the extracellular domain of TASK-2 between the first transmembrane segment and pore region by mutation to the neutral amino acids glutamine (Q) and asparagine (N).

Wild-type (WT) murine TASK-2 cDNA was subcloned into the bicistronic vector pIRE5-CD8 (Invitrogen). Single point mutations were generated by a PCR-based mutagenesis approach and confirmed by automated fluorescence sequencing (Lark). Chinese hamster ovary (CHO) cells were transfected with either WT or mutant plasmid using Fugene transfection reagent (Roche). Twenty-four to 72 h post-transfection CHO cells expressing the CD8\textsuperscript{+} antigen were positively identified by incubation with immunomagnetic particles coated with anti-CD8 antibody (Dynal) and subjected to whole-cell patch clamp analysis. Whole-cell currents were recorded in mammalian Ringer solution containing (mm): NaCl 145, KCl 5, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2, Heps 5, Pipes 5, titrated to between pH 5.8 and 8.8 with...
KOH or HCl. Currents were normalized with respect to those at pH 8.8 and Kd values calculated with a single binding site model. Results are given as means ± S.E.M. and statistical significance tested using Student’s unpaired t test.

The Kd for WT channels was pH 7.47 ± 0.08 (n = 9). The Kd values of mutant channels E28Q, K32N, K35N and K47N were all significantly lower than WT (P < 0.05); 7.12 ± 0.09 (n = 9), 7.20 ± 0.06 (n = 9), 7.27 ± 0.04 (n = 14) and 7.24 ± 0.06 (n = 12) respectively. The decrease in Kd implies a reduction in the pH sensitivity of the mutant channel. The Kd value for mutant channel H44N was not significantly different from WT; 7.43 ± 0.08 (n = 11).

The large extracellular domain of TASK-2 comprises approximately 50 amino acids, 13 of which are charged and therefore titratable by protons. Amongst these charged residues are five that are unique to TASK-2. The mutation of four of these charged residues to neutral amino acids resulted in a decrease in pH sensitivity. The exact mechanism of this reduction is unknown. The residues may themselves be titrated by protons or contribute to the effective concentration of protons at the pH sensor. In conclusion, three charged amino acids in the large extracellular domain contribute to the pH sensitivity of TASK-2.

Morton MJ et al. (2002). J Physiol 544, 103P.

This work was supported by the Wellcome Trust.

PC17

Effect of hypoxia on L-arginine transport in human fetal endothelium: role of protein kinase C and endothelial nitric oxide synthase

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Fetal hypoxia has been associated with intrauterine growth restriction, a disease that reduces mRNA levels for human cationic amino acid transporters 1 (hCAT1) and 2B (hCAT2B), but increases eNOS mRNA level in human umbilical vein endothelial cells (HUVECs) (Casanello & Sobrevia, 2002), and activates protein kinase C (PKC, Langdown et al. 2001). We studied the involvement of PKC and nitric oxide (NO) in the modulation of L-arginine transport by hypoxia in HUVECs.

Cells isolated from normal pregnancies (Ethics Committee approval and informed patient consent were obtained) were cultured in medium 199 (containing 20% bovine sera, 3.2 mM l-glutamine), and exposed (24 h) to normoxia (21% O2, 5% CO2, 93% N2), p-Amino acid transporters (P3C): l-arginine (21% O2, 5% CO2, 93% N2), l-arginine (21% O2, 5% CO2, 93% N2). l-Arginine transport (l-3H)l-arginine, 36.1 Ci mmol−1, 7.5–100 μM, 2 μCi ml−1, 37°C, 1 min) was determined in the absence or presence (30 min to 24 h) of S-nitroso-N-acetyl-l,L-penicillamine (SNAP, 100 μM, 30 min, NO donor), calphostin C (100 nM, PKC inhibitor), 13-acetate, 12-myristate phorbol ester (PMA, 100 nM, PKC activator) or Nω-nitro-l-arginine methyl ester (l-NAME, 100 μM, eNOS inhibitor). hCAT1, hCAT2B and eNOS mRNAs were amplified by reverse transcriptase-polymerase chain reactions. Gene expression was also assessed on cDNA microarrays (Clontech). eNOS activity was determined by l-[3H]l-citrulline formation from l-[3H]l-arginine (4 μCi ml−1, 30 min) and eNOS protein was detected by Western blot.

Hypoxia reduced the Vmax for L-arginine transport (2.8 ± 0.2 vs. 5.1 ± 0.3 pmol (μg protein)−1 min−1, n = 8, P < 0.05, means ± s.e.m., Student’s unpaired t test), with no significant changes in the apparent Km (normoxia = 149 ± 15 μM, hypoxia = 125 ± 12 μM). Hypoxia reduced the hCAT1 (50 ± 2.6%) and hCAT2B (52 ± 0.2%) mRNA levels, but increased eNOS mRNA (46 ± 2%) and protein (1.4-fold) levels. L-Citrulline synthesis was reduced (55 ± 4%) in hypoxia. Hypoxia-induced inhibition of L-arginine transport was reversed by SNAP or calphostin C. Hypoxia increased PKCα/βII activity (2.7-fold) and PKCz gene expression (12-fold).

In summary, hypoxia-induced inhibition of the L-arginine/NO pathway could be due to reduced hCAT1 and hCAT2B transporter expression, an effect that may involve PKC and NO.


This work was supported by FONDECYT (1030607, 1030781, 1000354 & 7000354), DIUC-University of Concepción (201.084.003-1.0) (Chile) and The Wellcome Trust (UK).

All procedures accord with current local guidelines and the Declaration of Helsinki.

PC18

Regulation of System A transporter 2 (SAT2) by ceramide in rat skeletal muscle cells

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The SAT2 System A transporter is one of the most highly regulated Na+-coupled transport mechanisms for small neutral amino acids (AAs) in mammalian cells and hence one most likely to have an active influence on cellular AA metabolism (Broer, 2002). The transporter is regulated at multiple levels and by many factors, including hormonal and nutritional stimuli that can modulate both its subcellular distribution and cellular expression (Hyde et al. 2001, 2002), and by changes in pH and electrochemical gradients that can alter transporter activity at the plasma membrane (Chaudhry et al. 2002). The sphingolipid ceramide (Cer) has been implicated in the pathogenesis of insulin resistance and apoptosis, because of its widely accepted role in tumour necrosis factor α signalling (Mathias et al. 1998). Since ceramide antagonises insulin action, we sought to determine whether it affects SAT2 function in L6 muscle cells, which is known to be regulated by insulin.

Cer induced a time- and dose-dependent decrease in System A activity, with maximal (~70%) reduction after 2 h of treatment with 100 μM Cer, whereas an inactive Cer analogue had no effect. This response was associated with a decrease in SAT2 protein in the plasma membrane, although no reduction in total cellular SAT2 content was observed. Protein synthesis and the uptake of leucine and glucose were largely unaffected by Cer, but significant changes in the intracellular AA profile for both System A and non-System A substrates was observed. Anaerobic stimuli such as insulin and leucine stimulated System A activity by up to 80% following a 30 min and 3 h incubation, respectively. System A transport was also enhanced (~2-fold) upon depriving cells of AAs for 4 h. The responses to both insulin and leucine were lost
upon preincubation of L6 cells with Cer, whereas the increase in System A activity in response to AA withdrawal was unaffected. Our results indicate that Cer suppresses transport of AAs via System A by promoting internalisation of SAT2 transporters at the plasma membrane and by desensitising the carrier to the effects of various stimuli. Since Cer levels are elevated in muscle during insulin resistance, the effects of the lipid on SAT2 may have clinical importance in Type II diabetes. Also, since System A is upregulated during cellular proliferation, the effects of pro-apoptotic compounds, such as Cer, on SAT2 may also have a role in regulated cell death.


This work was supported by the BBSRC, MRC and Diabetes UK.

**PC19**

The role of different types of Cl− channels in MDCK cyst development and growth

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Polycystic kidney disease is characterised by the massive enlargement of fluid-filled epithelial cysts that disrupt kidney function. Hanaoka et al. (1996) demonstrated that the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel plays a key role in fluid accumulation within the lumen of some, but not all, cysts from patients with polycystic kidney disease. This suggests that other types of Cl− channels might play an important role in cyst development and enlargement.

Using MDCK type I cells that express CFTR, we previously demonstrated that CFTR-driven fluid secretion increases the size of MDCK cysts (Li & Sheppard, 2002). In this study, we investigated the role of Ca2+-activated and volume-sensitive Cl− channels in cyst formation and growth. We began by assessing the function of Ca2+-activated and volume-sensitive Cl− channels in MDCK cells using the iodide efflux assay (for Methods, see Lansdell et al. 1998). To stimulate Ca2+-activated and volume-sensitive Cl− channels, we used ionomycin (1 μM) and a 50% hypotonic solution, respectively; to activate CFTR Cl− channels, we used forskolin (10 μM). In type I MDCK cells, forskolin, ionomycin and hypotonicity all stimulated an efflux of iodide and the magnitude of iodide efflux decreased in the rank order: hypotonicity (82 ± 9 nmol min−1) > ionomycin (18 ± 2 nmol min−1) = forskolin (14 ± 3 nmol min−1; means ± S.E.M.; n = 6 for all values). In contrast, in type II MDCK cells that lack CFTR, only hypotonicity stimulated an efflux of iodide. However, the magnitude of the response was drastically smaller than that stimulated by hypotonicity in type I MDCK cells (10 ± 1 nmol min−1; n = 6).

For cyst growth studies, we used cysts grown either in collagen gels or culture dishes for 6 days. Type I MDCK cysts formed using both substrates. However, type II MDCK cysts only formed in culture dishes. In the absence of drugs, a few large type I cysts with well-defined walls formed in culture dishes in the absence of drugs. In collagen gels, forskolin (10 μM) and ionomycin (1 μM) stimulated the formation and growth of type I MDCK cysts, but were without effect on type II MDCK cysts. The number and size of cysts grown in the presence of ionomycin (1 μM) were significantly smaller than those grown in the presence of forskolin (10 μM; P < 0.01; Student’s unpaired t test). A 50% hypotonic solution stimulated the growth of both type I and type II MDCK cysts. Interestingly, cysts grown in the presence of a 50% hypotonic solution developed into a solid mass of cells after 2–4 days. We interpret these data to suggest that the CFTR Cl− channel plays a dominant role in MDCK cyst formation and growth.


This work was supported by the NKRF.

**PC21**

Isolation and functional characterisation of rat PAT2 (proton-coupled amino-acid transporter 2)

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The H+–coupled amino acid transporter PAT1 has been cloned from rat (Sagné et al. 2001), mouse (Boll et al. 2002) and human (Chen et al. 2003). Recently, a related transporter (PAT2) was cloned from mouse, and characterised functionally following expression in Xenopus laevis oocytes (Boll et al. 2002). Here we report the cloning and functional characterisation of rat PAT2.

A rat lung cDNA library was screened using a probe designed using the mouse PAT2 cDNA sequence (Boll et al. 2002). X. laevis were killed humanely and oocytes removed for expression studies. [3H]Amino acid (proline, alanine, glycine, MeAIB, leucine and lysine, all 100 μM, 5 μCi ml−1) uptake (23°C, 40 min, pH 5.0–7.4) was determined both in the presence and absence of extracellular Na+ in X. laevis oocytes 3 days after injection with 50 ng rat PAT2 cRNA.

A full-length 2396 bp cDNA (including polyA tail) was isolated from a rat lung cDNA library. The cDNA encodes for a transporter protein (rat PAT2), 481 amino acids in length. At the amino acid level rat PAT2 has 93% identity with mouse PAT2 (Boll et al. 2002) and 75% (over amino acids 52–477) with rat PAT1 (LYAAT-1) (Sagné et al. 2001). Proline uptake (pH 5.0–7.4) in rat PAT2 expressing X. laevis oocytes was greater than that in water-injected controls (P < 0.001, ANOVA), uptake being pH dependent and significantly larger (P < 0.001) at pH 5.5 (71.67 ± 6.62 pmol oocyte−1 (40 min)−1 (20); mean ± S.E.M. (n) than at pH 7.4 (35.25 ± 2.21 pmol oocyte−1 (40 min)−1 (19)). Uptake was Na+ independent at both pH 5.5 and 7.4 (P > 0.05; Na+ versus Na−free conditions). Compared to PAT1, rat PAT2 is a high affinity transporter, KNa of proline of 0.20 ± 0.04 mM. Measurement of uptake of a number of radiolabelled amino acids suggests that glycine, alanine and MeAIB are also substrates for rat PAT2 whereas leucine and lysine are not.

In conclusion, rat PAT2 was cloned from a rat lung cDNA library and functions as a high affinity pH-dependent, H+–coupled, Na+–independent amino acid transporter. The physiological role of this transporter requires further investigation.


This work was supported by the MRC (G9801704), BBSRC (13/D17277) and NIH (HL64196 & AI49849).

*All procedures accord with current local guidelines.*