Sodium sensitive and insensitive copper accumulation by isolated intestinal cells of rainbow trout, *Oncorhynchus mykiss*

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The pathway(s) for copper (Cu) uptake across the mucosal membrane into intestinal cells has not been elucidated, and may include Cu uptake by the Cu-specific pathway encoded by Ctr1 or via Cu uptake through epithelial sodium channels (ENaCs). Our laboratory has developed a model of intestinal Cu absorption using a comparative physiological and genomic approach (Handy et al., 2000; Handy et al. 2002). In this study we aimed to explore whether or not Cu entry into intestinal cells was Na+-sensitive. Copper accumulation in freshly isolated intestinal cells from rainbow trout was measured by ICP-AES after exposure to 0-800 µM CuSO4 for 15 minutes. Cu accumulation by cells was 1.88 ± 0.52 compared with 0.05 ± 0.01 nmol Cu/mg cell protein/h with external Cu ([Cu]o) of 800 µM and no-added Cu, respectively (mean ± S.E.M., n = 6; Kruskal Wallis, P<0.05). Deduction of a rapid Cu accumulation on/in cells at time zero (about 12% of the total Cu when [Cu]o was 800 µM) revealed a saturable uptake curve which reached a plateau at 400 µM Cu (Km, 216 µM Cu; Vmax, 1.09 nmol Cu/mg cell protein/h, 140 mM NaCl throughout). Incubating cells at 4°C did not stop Cu accumulation. Lowering external Na+ to 11 mM (low Na+o) generally decreased Cu accumulation into the cells over 15 min. In low Na+o conditions Cu accumulation was exponential (non-saturable). Na+-insensitive Cu accumulation dominated (59% of total Cu accumulation) when [Cu]o was 400 µM or less. At high Cuo (800 µM), removal of Na+ caused a 45% increase in Cu accumulation. Pre incubation of cells with epithelial Na+ channel (ENaC) blocking agents for 15 minutes (normal NaCl throughout) caused Cu accumulation to increase by 40 fold (100 µM phenamil), 21 fold (10 µM CDPC) or 12 fold (2 mM amiloride) when Cuo was 800 µM compared to drug-free controls (all significantly different from drug-free controls, Kruskal Wallis, P<0.05). Lowering external chloride (Cl−o) from 131.6 to 6.6 mM (replaced by Na+ gluconate) caused Cu accumulation to increase 11 fold when Cuo was 800 µM. Application of 0.1 mM DIDS (normal Cl−o) caused a similar effect. Lowering external pH from 7.4 to pH 5.5 produced a 17 fold, saturable, increase in Cu accumulation which was not explained by increased instantaneous Cu accumulation on/in cells at low pH. We conclude that Cu accumulation by intestinal cells is mainly Na+-insensitive and more characteristic of a Ctr1-like pathway than Cu uptake through ENaCs.


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Where applicable, the experiments described here conform with Physiological Society ethical requirements.
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C3
An electrogenic V-H+-ATPase drives electrolyte transport in the isolated perfused larval Drosophila midgut.
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Early third-instar larvae of wild-type Drosophila feed actively. The epithelium lining the gut is a single layer of cells, and is therefore an attractive preparation for study of nutrient and fluid and electrolyte transport. As there is considerable variation in the dimensions, cell types and transport functions along the distinct segments of the gut, we have developed an in vitro method for microperfusion of individual gut segments that enables independent control of solute composition of luminal and hemolymph compartments with simultaneous measurement of transepithelial potential (V_t) and resistance (R_t) and basolateral pH. Bilateral microperfusion of individual gut segments that enables independent control of solute composition of luminal and hemolymph compartments with simultaneous measurement of transepithelial potential (V_t) and resistance (R_t) and basolateral intracellular potential (V_i) and intracellular potential with ion-selective microelectrodes. Midgut segments of 1 to 1.5mm length, lumen diameter 170µm and outer diameter 200um, were dissected and perfused with isotonic insect Ringer at 23 °C. Details of the basic perfusion technique with glass micropipettes and measurement of electrical parameters have been described earlier (Tripathi & Boulpaep, 1988). Data are presented as means ± SEM and compared by Student’s t-test using paired data.

Cannulation of one end only yielded a lumen negative V_t of -17.8±0.4 mV (n=94). Simultaneous cannulation of both ends increased V_t to ~56.8±1.0 mV (n=94). R_t measured by terminated cable analysis was 719±34 Ω.cm² (n=26); the epithelium is therefore tighter than most tubular epithelia e.g. proximal renal tubules. V_t was -48.8±1.8 mV (n=58) and indicates that the apical membrane potential is of the order of 8 mV. Bilateral substitution of Cl with Glucosate increased V_t from -46.5±3.1 to -54.5±3.7 mV (n=8; p<0.005) and increased R_t from 822±68 Ω.cm² to 2452±340 Ω.cm² (n=8; p<0.0001). Bilateral substitution of Na with Na-glucose or Na-glucosate increased R_t from 641±71 Ω.cm² to 2313±535 Ω.cm² (n=4; p<0.05). Bilateral replacement of Cl with Na or HCO,Cl replacement with HEPES were without significant effects.
Bafilomycin A1 (a V-H-ATPase inhibitor) added to the bath to a concentration of 750 nM reduced V_t from -27.4±1.2 to -21±1.6 mV (n=3; p<0.005) without a significant increase in R_t. The inhibitory effect of bafilomycin was dose dependent and became significant at a concentration of 1µM. At 7.5µM transepithelial current was inhibited ~65% (control 59±8 µA.cm⁻² to 21±2 µA.cm⁻² (n=5; p<0.001).

We conclude that the secretion of base into the lumen by this segment is accomplished by an electrogenic V-ATPase extruding H⁺ to the hemolymph and is the primary driving force for electrolyte transport. The high Cl and Na permeability permits net solute transport which would also result in net water flow across the epithelium.
Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C4
Regulation of intestinal (Caco-2) dipeptide uptake by phosphodiesterase inhibitors
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The intestinal di/tripeptide transporter hPepT1 plays an important role in the absorption of dietary protein and peptidomimetic drugs (Rubio-Aliaga & Daniel, 2002). For optimal absorptive transport via hPepT1 an active Na⁺/H⁺ exchanger (NHE3) is required at the brush border membrane (Thwaites et al. 2002). NHE3 is activated by the decrease in pH associated with hPepT1-mediated H⁺/dipeptide swap. NHE3 extrudes protons and thus maintains the driving force (the transmembrane H⁺ electrochemical gradient) required for further H⁺/dipeptide transport. NHE3 inhibition either by selective pharmacological inhibitors (e.g. S1611) or through activation of the cAMP/protein kinase A pathway (e.g. by vasoactive intestinal peptide) reduces hPepT1 activity indirectly via a reduction in the driving force (Anderson et al. 2003). The aim of this study was to investigate the effects of various phosphodiesterase inhibitors (which raise intracellular cAMP and cGMP levels by inhibiting their breakdown) on hPepT1 function. [¹⁴C]Gly-Sar uptake (0.5µCi.ml⁻¹, 100µM) across the apical membrane of human intestinal Caco-2 cell monolayers (passage 100-116, 14-19 days post-seeding) was measured over 15min (apical pH 6.5, basolateral pH 7.4) in the presence and absence of various phosphodiesterase inhibitors. Caffeine and theophylline reduced Gly-Sar uptake in a concentration-dependent manner. 5mM caffeine reduced Gly-Sar uptake [mean ± SEM (n)] from 684±19 to 351±11 (17-18) pmol.cm⁻².15min⁻¹ (p<0.001, ANOVA, Bonferroni post test). In contrast, caffeine and theophylline (5mM) did not inhibit Gly-Sar uptake under conditions where NHE3 would be inactive e.g. in the absence of extracellular Na⁺, no difference (p>0.05) in uptake was observed (242±49 and 242±10 (17-18) pmol.cm⁻².15min⁻¹ in the absence and presence of caffeine (5mM), respectively. Pentoxifylline is a phosphodiesterase inhibitor given orally to treat intermittent claudication (Jocoby & Mohler, 2004). Pentoxifylline (5mM) inhibited Gly-Sar uptake by reducing Vmax [from 8246±913 to 5369±1167 (6) pmol.cm⁻².15min⁻¹ (p<0.05, Student’s t-test) without effect on affinity [Km 1.0±0.2 and 1.6±0.6 (6) µM, (p>0.05, Student’s t-test) in the presence and absence of pentoxifylline, respectively]. These observations suggest that hPepT1-mediated absorption of any drug may be reduced (following NHE3 inhibition) by phosphodiesterase inhibitors from diet or those co-administered as therapeutic agents.
C5

Vigabatrin transport across the intestinal (Caco-2) apical membrane is via the human H+-coupled amino acid transporter (hPAT1)

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The GABA-transaminase inhibitor, gamma-vinyl-GABA (vigabatrin), has 100% oral bioavailability and is used orally to treat some forms of epilepsy (Goldsmith & de Bittencourt, 1995; Richens, 1992). There are no reports on the mechanisms involved in vigabatrin transport across the small intestinal wall. We have recently identified that the H+-coupled amino acid transporter (system PAT), characterised functionally using human intestinal Caco-2 cell monolayers (Thwaites et al. 2000), represents the classical “imino acid carrier” in both human and rat small intestine (Anderson et al. 2004). A system-PAT-related cDNA clone was isolated from a Caco-2 cell cDNA library and named hPAT1 for human Proton-coupled Amino acid Transporter 1 (Chen et al. 2003). The purpose of this investigation was to determine the role of system PAT/hPAT1 in vigabatrin absorption. Radiolabelled uptake experiments (3H]-GABA, 20-100μM, 0.5-5.0μCi.mL-1) were performed using Caco-2 cell monolayers (passage no. 108-118, 14-17 days post-seeding, 15min at 37°C) and hPAT1-expressing Xenopus laevis oocytes (cRNA 50ng.oocyte-1, 2 days post-injection, 40min at 23°C). Under optimal transport conditions (pH 5.5, Na+-free buffers) vigabatrin (10μM) caused a significant reduction in [3H]-GABA uptake by 55.8±5.0% (mean ± SD, n=5 oocytes; p<0.001, ANOVA Bonferroni post-test). Inhibition of [3H]-GABA uptake via unlabelled GABA and vigabatrin had a similar concentration dependence producing IC50 values of 2.1±1.4 and 2.8±1.6 mM, respectively. In hPAT1-expressing oocytes vigabatrin reduced [3H]GABA uptake by 55.8±12.3 (9%) %. To determine whether vigabatrin was a transport inhibitor or substrate, substrate-induced current flow into hPAT1-expressing oocytes was measured under voltage-clamped conditions, where both GABA and vigabatrin (both 10μM) induced similar current flow (the response to vigabatrin was 75.7±6.9 (3%) % (mean ± SD) of that with GABA). Similar observations were made using the GABA analogues TACA (transaminocrotonic acid) and guvacine. No current was observed in water-injected oocytes. In conclusion vigabatrin is transported across the apical membrane of Caco-2 cell monolayers via the high capacity, H+-coupled transporter hPAT1, which emphasises the potential of this transporter as a route for oral drug delivery.


Cl− secretion induced by Ca2+ agonists is impaired in distal colon of a Kcnm4 null mouse

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Ca2+-dependent anion secretion in distal colon requires the simultaneous activity of apical CFTR Cl− channels activated by cAMP and basolateral (BL) K+ channels activated by Ca2+. Three classes of Ca2+-activated K+ channels have been distinguished from their single channel conductances. The small-, intermediate-, and large-conductance channels SK, IK, and BK, respectively. SK channels are encoded by three genes, SK, IK, and BK, respectively. The small-, intermediate- and large-conductance channels SK, IK, and BK, respectively.

Ca2+-activated K+ channels of all three types have been identified in the colon and could participate in Ca2+-dependent anion secretion. We have now used an animal deficient in IK1, the Kcnm4 null mouse (Begenesich et al., 2004), to test for its participation in this process.

A piece of distal colon was gently stripped of the muscle layer, placed in an Ussing chamber (0.1 cm2) in a bicarbonate buffered solution, gassed with 95% O2/5% CO2 at 37°C. The transepithelial potential difference was monitored continuously and 20 µA pulses were passed to calculate tissue resistance and equivalent short circuit current (Isc). To induce cAMP-mediated Cl− secretion tissues were incubated with 100 µM IBMX and 1 µM forskolin. Chromanol 293 B (10 µM) was added at the BL side to close KCNQ1/KCNE3 K+ channels. BL carbachol (CCh, 100 µM) was used as Ca2+ agonist. Clotrimazol (3 µM) and Ba2+ (5 mM) were used as K+ channel blockers. For K+ secretion experiments, tissues were incubated with indomethacin (2 µM) prior to CCh application, to avoid basal release of prostaglandin and consequent cAMP increase.

Wild type (WT) and knockout (KO) animals had cAMP-induced Cl− secretion of similar magnitude (ΔIsc = -149 ± 21 and -144 ± 11 µA cm-2, means ± SEM, n=5 & 4 respectively). When CCh was added, only WT tissues had a current response consistent with Ca2+- dependent Cl− secretion (ΔIsc = -174 ± 12 µA cm-2), which was decreased by the IK1 inhibitor clotrimazol (ΔIsc = -38 ± 3.6 µA cm-2, n=5, p<0.0005). In KO tissues, CCh induced positive Isc change (ΔIsc 17 ± 6 µA cm-2, n=4) consistent with K+ secretion, which was inhibited by apical Ba2+ (ΔIsc 8.7 ± 1.8 µA cm-2, n=3; p<0.05), but not by clotrimazol (ΔIsc 19 ± 7 µA cm-2, n=3). Experiments with epithelia treated with indomethacin suggest that both WT and KO tissues are able to secrete K+ under CCh stimulation. This was inhibited by Ba2+ but not affected by clotrimazol.

Our data suggests that IK1 encoded by the Kcnm4 gene is the main Ca2+-activated K+ channel that drives Cl− secretion by Ca2+-agonists in mouse distal colon. They also suggest that a different, as yet unidentified Ba2+-sensitive apical K+ channel mediates CCh-activated K+ secretion.


CAF is a PhD student at the Universidad Austral de Chile.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.
The effect of phenformin on short-circuit current (Isc) across H441 human lung epithelial cells

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Active reabsorption of Na⁺ across the alveolar epithelium is essential for lung fluid homeostasis. Na⁺ entry at the apical membrane of the lung epithelial cell is predominantly via the amiloride-sensitive Na⁺ channel (ENaC) down its electrochemical gradient. This gradient is generated and maintained by Na⁺ extrusion via the Na⁺K⁺ATPase pump located at the basolateral membrane.

Phenformin, an oral hypoglycaemic biguanide has been shown to influence cellular metabolism and membrane function. This has recently been attributed, in part, to its ability to activate AMP–activated protein kinase (AMPK) (Sakamoto et al. 2004). Therefore, we explored the effect of phenformin and activation of AMPK on ion transport across H441 human lung epithelial cells.

H441 cells were cultured on permeable supports for 7 days and monolayers were treated both apically and basolaterally with 10mM phenformin for 1 hour. Monolayers were then mounted in Ussing chambers where phenformin was circulated in physiological saline throughout the course of the experiment. Spontaneous short circuit current (Iₛₖ) was measured by clamping transepithelial voltage (Vₜ) at zero. Statistical analysis was carried out using Student’s t-test where p values of < 0.05 were considered significant. Data are presented as mean ± SEM.

Treatment with phenformin resulted in a significant decrease in spontaneous Iₛₖ (18.9 ± 2.29 μA.cm⁻²) compared to that of controls (42.3 ± 1.8 μA.cm⁻², p < 0.001, n = 4). Forskolin (10μM) induced a rise in Iₛₖ of 13.8 ± 4.2 μA.cm⁻² in control cells which was not evident in the presence of phenformin (0.2 ± 0.32 μA.cm⁻² p < 0.05, n = 3). Application of 10μM amiloride to the monolayers showed that phenformin significantly reduced the amiloride-sensitive component of Iₛₖ (14.3 ± 1.3 μA.cm⁻²) compared to control (46.9 ± 4.9 μA.cm⁻² p < 0.01, n = 3). Na⁺K⁺ATPase pump capacity was determined by apical permeabilisation with 75μM nystatin followed by blockade with 100μM ouabain. Ouabain-sensitive current was also significantly reduced in phenformin treated cells (12.3 ± 0.3 μA.cm⁻²) compared to that of controls (39.4 ± 6.2 μA.cm⁻² p = 0.01, n = 3). Phenformin induced a 3.8-fold rise in AMPK activity in H441 cells from 0.5 ± 0.1 to 1.8 ± 0.2 nmol min⁻¹ mg⁻¹ (p = 0.001, n = 4). Taken together these data indicate that 10mM phenformin suppresses amiloride-sensitive Na⁺ transport across H441 cells via a pathway that includes activation of AMPK and inhibition of Na⁺K⁺ATPase.


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Fig. 1. Dexamethasone-treated (0.2 µM) H441 cells grown on glass coverslips were held under voltage clamp in the perforated patch recording configuration. The membrane currents were evoked by driving $V_m$ through a series of 7 steps (-90 mV to +68 mV; holding potential -40 mV) every 15 s and A shows the effects of thapsigargin upon the currents recorded at each such potential; Ca$^{2+}$ was removed from the external solution as indicated. B Whole cell current voltage relationship (mean ± s.e.m., n = 9) in control and thapsigargin-treated cells.


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Increased transepithelial Na⁺ transport due to the activation of an intermediate conductance K⁺ (Iₖ) channel in H441 airway epithelial cells.

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Thapsigargin evokes increased Na⁺ transport in H441 cells and, at least in part, this response is due to a Ca²⁺-dependent increase in K⁺ conductance (Gₖ) (McNeill et al., 2005). To characterise the channels underlying this response, we have now explored the effects of putative K⁺ channel blockers upon the thapsigargin-evoked increase in membrane current. Ba²⁺ (5 mM) caused 73 ± 8 % inhibition whilst 1 μM clotrimazole essentially abolished the response (97 ± 5 %) and, since apamin (1 μM, n = 4) and iberiotoxin (0.1 μM, n = 7) were ineffective, this suggests that the thapsigargin-evoked increase in Gₖ (McNeill et al., 2005) reflects increased activity of K⁺ channels belonging to the Iₖ family. Further evidence of this came from RT-PCR based studies which showed that H441 cells expressed Iₖ channel mRNA transcripts but did not express either the large or small conductance K⁺ channel. Moreover, 1 mM EBIO, a substance that characteristically activates Iₖ channels, caused an increased in membrane conductance (control: 353 ± 68 pS cell⁻¹, EBIO: 1222 ± 245 pS cell⁻¹) that reflected activation of a K⁺ selective current and which caused a hyperpolarization of Vₘ (control: -52 ± 3 mV; EBIO: -79 ± 1 mV, P < 0.001). These data suggest that the thapsigargin-evoked increase in Iₖ (McNeill et al., 2005) is due to increased activity of Iₖ channels, a hypothesis that predicts that EBIO-evoked activation of these channels will mimic the electrometric response to thapsigargin. We therefore explored the effects of EBIO upon Iₖ and [Ca²⁺], measured simultaneously in polarised H441 cells (Fig 1). As anticipated, EBIO increased Iₖ without affecting [Ca²⁺], and so the present data suggest that Ca²⁺ mobilising agonists could be important regulators of epithelial Na⁺ transport in some tissues.
C124

Mechanism of thromboxane A₂-stimulated cell proliferation of human colorectal cancer cells

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Tumor growth of colorectal cancers accompanies up-regulation of inducible cyclooxygenase-2, which catalyzes a key step in conversion of arachidonic acid to prostaglandin H₂ (PGH₂) (Marx, 2001). In the downstream of COX, thromboxane synthase (TXS) catalyzes the conversion of PGH₂ to thromboxane A₂ (TXA₂). We found recently that TXS is significantly up-regulated in the human colorectal carcinomas (Sakai et al. 2003). TXS was also highly expressed in human colorectal cancer cell lines. Disruption of TXS protein by the antisense oligonucleotide inhibited the proliferation of the cancer cells (Suzuki et al. 2004). Herein we investigated if the Kᵥ7.1 (KᵥLQT1), a voltage-dependent K⁺ channel, is involved in the TXA₂-induced cell proliferation. Human colorectal cancer tissue and its accompanying normal mucosa were obtained from surgical resection of Japanese patients in accordance with the recommendations of the Declaration of Helsinki and with the ethics committee approval. The expression of Kᵥ7.1 protein in human tissues and human colonic carcinoma cell lines was examined by Western blotting. Cell proliferation assay was performed by counting the number of the cell in a 12-well plate. Results are shown as means ± S.E.M. The data were statistically analysed using one-way ANOVA and Tukey’s multiple comparison test. We compared the expression levels of Kᵥ7.1 protein between human colorectal cancer tissue and its accompanying normal mucosa. It was found that Kᵥ7.1 is up-regulated in human cancer tissues (70 kDa; 10 of 10 cases, 100%). Significant expression of Kᵥ7.1 protein was also observed in the human colorectal cancer cell lines such as KM12-L4, HT-29, T-84, WiDr (n = 3). A stable analog of TXA₂ (STA₂; 0.1 µM) stimulated the proliferation of KM12-L4 cells from (1.3 ± 0.03) x 10⁶ to (1.65 ± 0.04) x 10⁶ cells (n = 5, P < 0.01), and the STA₂-induced effect was completely inhibited by chromanol 293B (10 µM), an inhibitor of Kᵥ7.1 (n = 5, P < 0.01). STA₂ (0.1 µM) significantly increased the expression level of Kᵥ7.1 protein by (2.09 ± 0.09)-fold in the KM12-L4 cells (n = 4, P < 0.01). In the cells, STA₂ also increased the chromanol 293B-sensitive K⁺ current from 8.87 ± 1.01 to 16.91 ± 1.25 pA pF⁻¹ at +30 mV (n = 7, P < 0.01). These results suggest that paralleled up-regulations of TXS and Kᵥ7.1 channel may be involved in the tumor growth of human colorectum.


C125

Low extracellular pH protects mouse macrophages against apoptosis induced by oxidised low density lipoprotein under normoxic or hypoxic conditions

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Atherosclerotic lesions are hypoxic (Bjornheden et al. 1999) and have a low extracellular pH (Naghavi et al. 2002) (probably in coexistence) compared to the normal intima of an artery. Oxidised low density lipoprotein (LDL) is believed to be involved in atherosclerosis and apoptosis of macrophages occurs in atherosclerotic lesions. We have therefore investigated the effects of pH and hypoxia on apoptosis induced by oxidised LDL in macrophages. Human LDL was oxidised by dialysis for 24 h against 10µM CuSO₄ at 37°C and contained high levels of oxysterols and only low levels of lipid hydroperoxides (as they had largely decomposed). Murine J774 macrophages were cultured in the absence or presence of oxidised LDL (100µg protein/ml) at 3% oxygen (hypoxia) or 20% oxygen (normoxia for cell cultures) at pH 7.4 or 7.0 for 18 h. Flow cytometry was used to measure apoptosis (annexin V binding) and necrosis (propidium iodide uptake). Oxidised LDL was damaging for most cells, causing a large increase in apoptosis and secondary necrosis (apoptosis followed by necrosis) (annexin V binding) and necrosis (propidium iodide uptake). Low extracellular pH had a marked protective effect against cell death induced by oxidised LDL (decreasing apoptosis plus secondary necrosis) under both oxygen levels (78.5±6.0% at pH 7.4 vs. 44.8±5.3% at pH 7.0 for normoxia; mean ± SEM, n = 17 individual experiments, P<0.001). The healthy cell population increased from 15.8±3.7% at pH 7.4 to 52.2±5.2% at pH 7.0 (P<0.001). Similar effects were observed when the extracellular pH was lowered under hypoxic conditions. We conclude that low extracellular pH in atherosclerotic lesions may protect macrophages against the cytotoxic effects of oxidised LDL under both normoxic and hypoxic conditions.


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Role of neurotrophic factors (NTF) and extracellular matrix components (ECM) on VIP expression in the rat enteric nervous system (ENS)

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We studied changes in vasoactive intestinal peptide (VIP) and neuronal nitric oxide synthase (nNOS) expression by myenteric neurons during early postnatal development in parallel with maturation of the environment of myenteric neurons, specifically the expression of ECM components and NTF. Although these molecules are known to promote neuronal differentiation [Shetty & Turner, 1998; Sieber-Blum et al. 1981], very little is known about their role in the early postnatal ENS.

Sprague-Dawley rats were killed by cervical dislocation. The ileal muscularis externa, including myenteric ganglia, was processed for semi-quantitative RT-PCR at postnatal days P1, P7 and P21 (internal standards: βIII tubulin and PGP9.5). VIP (Fig. 1), nNOS and NT-3 mRNA levels showed a consistent increase between P1 and P21. Although no changes in GDNF mRNA levels were observed, there was an increase in levels of mRNA of its co-receptor, GFRA-1, indicating that the responsiveness of myenteric neurons to GDNF might be enhanced at older ages. Immunofluorescent labelling of ileal cross sections revealed changes in the levels of laminin (peak at P7) and fibronectin (peak at P21).

Next, we tested the effect of ECM and NTF on myenteric neurons in cultures of dissociated myenteric ganglia (P7/8) [Schafer et al. 1997]. Changes in cell numbers per unit area of coverslips were measured after double labelling for βIII tubulin and VIP. GDNF, but not NT-3 or BDNF (all at 1 ng/ml), enhanced survival of the neuronal population (35-67% increase, n=3 experiments, P<0.02, paired t-test) and the percentage of VIP positive neurons (39-59%, n=3, P<0.02). Elevation of VIP and nNOS mRNA levels after treatment with GDNF was confirmed by RT-PCR. The effect of GDNF on neuronal survival was maintained in the presence of laminin (26-58% increase, n=3, P<0.05) and fibronectin (34-55%, n=3, P<0.012), although neither factor alone influenced neuronal survival. Fibronectin (10-18% increase, n=3, P=0.10), but not laminin (19-81% increase, n=3, P<0.04), reduced the effect of GDNF on the proportion of VIP positive neurons.

Our study suggests that GDNF regulates VIP and nNOS expression by myenteric neurons. Fibronectin was found to influence the effect of GDNF on VIP phenotype. Together with the observed changes in the environment of myenteric neurons in the early postnatal gut, our results indicate that both NTF and ECM components are likely to contribute to postnatal development of the ENS.

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Fig. 1. Increase in VIP mRNA levels between P1-P21 (internal standard PGP9.5; L: Ladder).


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Can the Na⁺ pump mediate passive cation movements in the absence of ATP and P₂⁻?

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We have been using ATP analogues to try and map two ATP binding sites in the α subunit of pig kidney Na,K-ATPase [Cavieres, 2000; Ward & Cavieres, 1998, 2003]. In the native enzyme, TNP-8N₃[^αβ]-[αβ²P]-ADP anchors at αLys480, near the FITC site; the FITC-enzyme incorporates the radioactive analogue at a downstream site instead. Here we focus on the conditions for photoinactivating and labelling both native and FITC-modified Na,K-ATPase with TNP-8N₃-ADP, specially the effects of Na⁺ and K⁺. The native sodium pump can be photoinactivated by TNP-8N₃-ADP in the presence of just 50 mM Tris/Cl⁻ (pH 7.5), 1 mM EDTA. The rate constant (k[^kinact]) increases hyperbolically with k[^kinact(max)]=0.054±0.002 min⁻¹ and K[^D(TNP-8N₃-ADP)=0.11±0.02 μM (mean±S.E.); ATP and TNP-ATP protect with high affinities. Addition of Na⁺ does not affect k[^kinact] but K⁺ decreases it, with K[^D(Na⁺)=12±4 μM. Sodium ions reverse the K⁺ effect, and K[^D(Na⁺)=0.58±0.08 μM at 30 mMK⁺. All this is to be expected, as both native and FITC-modified Na,K-ATPase spontaneously adopt an E1 or "Na⁺ form", whilst K⁺ causes a conformational shift towards a "K⁺-occluded form", both cations acting at intracellular sites (Rephaeli et al., 1986). In the FITC-enzyme the surviving K⁺-phosphatase and P₂⁻-phosphorylation reactions cannot be photoinactivated in the presence of Tris alone, not even at 200-fold higher TNP-8N₃-ADP concentrations. In this case, Na⁺ promotes photoinactivation hyperbolically with similar k[^kinact(max)]=0.30±0.24 μM, but with K[^D(Na⁺)=97±9 mM; K⁺ counters this effect with K[^D(K⁺)=2.5±0.6 mM (Ward & Cavieres, 1998). As the intracellular Na⁺ and K⁺ affinities in the FITC-enzyme are similar to those in native Na,K-ATPase (Rephaeli et al., 1986), the latter Na⁺ and K⁺ effects can only be attributed to an external cation site. The hyperbolic Na⁺ activation may result from occupation of the "third external Na⁺ site" (Cavieres & Ellory, 1975).

It is then possible that Na⁺,K-ATPase can shuttle K⁺ (and Na⁺?) plus unloaded cation sites in the absence of ATP and P₂⁻; the forms that react with TNP-8N₃-ADP would be E₁ (or E₁Na⁺⁺) and E₂ Na⁺⁺ in the native and FITC-modified enzymes, respectively.
To be commensurate with $k_{\text{act(max)}}$, the slippage needs to be about 0.02% of the active transport rate.


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Where applicable, the experiments described here conform with Physiological Society ethical requirements.
Development of a novel method for the simultaneous measurement of free and conjugated bile acids in human serum from pregnant women with obstetric cholestasis

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Assessment of serum bile acids is central to the diagnosis of many liver disorders. The methods currently available are time consuming and do not allow the simultaneous measurement of a range of bile acids and their associated conjugates. The aim of this study was to develop a high performance liquid chromatography (HPLC) electrospray ionisation mass spectrometry (MS) technique to investigate bile acid metabolism in human serum from normal pregnant women and pregnant women with the liver disorder obstetric cholestasis (OC).

Bile acids profiles were investigated in fasted serum from pregnant women with OC (n=41) and normal healthy pregnant women (controls, n = 11) in the third trimester 37.6±1.3 and 39.9 ±1.4 weeks ± SD respectively. Informed consent was obtained. Comparisons were made using linear regression with robust standard errors and geometric means with 95% confidence intervals (CI).

A HPLC-MS technique was validated for the simultaneous evaluation of low and high abundance conjugated and unconjugated bile acids in human serum incorporating efficient resolution, high sensitivity, good reproducibility and a low detection limit (50pg). Total bile acids were found to be significantly elevated in the OC group (Table 1), however, more relevant information was gained by investigating the differential bile acid profile and state of conjugation. Table 1 details the most abundant bile acids in serum. We demonstrated that bile acids are predominately conjugated with glycine and taurine in the maternal circulation. In OC taurine conjugates predominate and primary bile acids in serum. We demonstrated that bile acids are predominately conjugated with glycine and taurine in the maternal circulation.

This method has been used to measure abnormal bile acid profiles in women with OC and will potentially be a useful tool for the assessment of serum bile acid profiles in other liver diseases. An improved evaluation of bile acid metabolism may provide better diagnostic or pathophysiological information in liver disease.

Table 1. Bile acid (BA) biochemistry.

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>OC n=41 Mean</th>
<th>Comparison P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total BA (enzymatic method) µmol/L</td>
<td>12.6 (9.8-15.2)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Sum BA µmol/L *</td>
<td>12.7 (10.1-16.0)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Taurocholic acid µmol/L *</td>
<td>4.6 (3.4-6.4)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Taurodeoxycholic acid µmol/L *</td>
<td>1.7 (1.3-2.4)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Glycodeoxycholic acid µmol/L *</td>
<td>1.1 (0.9-1.4)</td>
<td>P = 0.134</td>
</tr>
</tbody>
</table>

measured by HPLC-MS

This work was supported by Tommys’, the Baby Charity [No: 1060508], Lauren Page Trust and University of London Triangle Trust.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.
receptor-activated and voltage-operated Ca\(^{2+}\) channels, significantly reduced the peak contractile response to histamine (25 ± 9\%, \(p<0.005\), \(n=6\)) and also reduced initial resting tension (\(p<0.05\)). The selective sarcoplasmic reticulum Ca\(^{2+}\) pump inhibitor thapsigargin (1\(\mu\)M), used to deplete intracellular Ca\(^{2+}\) stores, had no effect on basal tone or on histamine-mediated contractions (\(n=5\)). In conclusion it would appear that histamine-induced contractions of chicken small intestine smooth muscle are heavily dependent on extracellular Ca\(^{2+}\) influx via receptor-activated Ca\(^{2+}\) channels. Intracellular Ca\(^{2+}\) release appears to have little role to play in histamine-mediated contractions. The basal tone of the tissue is both SK&F 96365 and nifedipine sensitive, suggesting that different Ca\(^{2+}\) channels may contribute to the maintenance of resting tone and histamine-mediated contractions.

This work was supported by the Higher Education Authority of Ireland.

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**PC34**

**Modulation of murine hepatic portal vein rhythmicity by food intake**

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The hepatic portal vein (HPV) is a rhythmically active blood vessel situated between the gastrointestinal tract and the liver that supplies 80% of the blood perfusing the liver. Whilst the generation of spontaneous activity by PVs from different species is well documented there is no information on the regulation of this activity by dietary factors. The present study investigated whether there was any influence of food intake on the spontaneous activity of HPV from adult babl-C mice. HPVs were harvested from fed animals or mice deprived of food for 16 h (fasted) and humanely killed. HPVs were mounted for isometric tension recording in standard tissue baths containing Krebs solution at 37\(^\circ\)C and aerated with 95%\(\text{O}_2\)/5%\(\text{CO}_2\). All tissues studied were spontaneously active within 10 mins of mounting. In HPVs from fed animals the activity exhibited a number of different contractions whereas in all HPVs from fasted animals only single contractions were recorded. The mean interval between contractions was significantly longer in HPVs from fasted animals (6.3 ± 0.2 s compared to 4.7 ± 0.12 s, mean ± SEM, \(p = <0.001\), data from 25 and 14 animals respectively). The duration of individual contractions was also briefer in HPVs from fasted mice (4.2 ± 0.09 s vs 5.74 ± 0.22 s, \(p<0.001\), Student's t test) and individual contractions were significantly smaller (0.1 ± 0.02 g compared to 0.18 ± 0.01, \(p <0.01\)). In comparison the effects of exogenously applied phenylepherine and 5-HT (100 \(\mu\)M) were not significantly different between both groups. However the maximal response to 60 mM KCl was smaller in HPVs from fasted mice (0.17 ± 0.02 g compared to 0.21 ± 0.02, \(p <0.05\), \(n=9\) and 14). These data show that food deprivation has a profound effect on PV rhythmicity.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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**PC35**

**Expression of KCC3 protein in gastric parietal cells**

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The K\(^+-\text{Cl}^-\) cotransporter (KCC) plays a significant role in the cell volume regulation, epithelial transport and ion homeostasis of many cell types (Hebert et al. 2004). So far, four KCC isoforms (KCC1-4) have been cloned. KCC1 and KCC4 are expressed ubiquitously. KCC2 is expressed in neurons in the central nervous system and retina. KCC3 is abundant in muscle, brain, spinal cord, kidney, heart, pancreas and placenta. So far, the expression of KCC isoforms in stomach has not been reported.

Here we tested whether KCC isoforms are expressed in rat, mouse, rabbit and human gastric mucosa. Rats and mice were humanely killed by cervical dislocation. Rabbits were humanely killed by the intraperitoneal administration of an overdose of urethane (2 g kg\(^{-1}\)). Isolated rabbit gastric glands were prepared as previously described (Sakai et al. 2003). Human gastric specimens were obtained from surgical resection of Japanese patients with gastric cancer in accordance with the recommendations of the Declaration of Helsinki and with the ethics committee approval. The expression of KCC mRNA and the protein in the samples was examined by Northern blotting and Western blotting, respectively. The distribution of KCC protein in the tissue sections was examined by immunohistochemistry.

Using isolated gastric mucosa and the gastric parietal cells of rats and rabbits, we compared the expression level of KCC1, 3, and 4 mRNAs between the mucosa and the parietal cells. Northern blot analysis showed that only KCC3 mRNA is more abundant in the parietal cells compared with the gastric mucosa. In rats, the expression level of KCC3 mRNA in the parietal cells was (4.7 ± 0.2)-fold higher than that in the gastric mucosa (\(n = 3, P < 0.01\)). In rabbits, the expression level of KCC3 mRNA in the gastric parietal cells was (2.0 ± 0.2)-fold higher than that in the gastric mucosa (\(n = 3, P < 0.05\)). Western blot analysis using a specific anti-KCC3 antibody showed the expression of KCC3 protein (~160 and ~190 kDa) in isolated gastric mucosa of rats, mice and humans, and in isolated rabbits gastric glands (\(n = 3-5\)). Immunohistochemistry in isolated mucosa of rats, mice and rabbits showed the expression of KCC3 protein in the parietal cells (\(n = 3-5\)). In human gastric cancer tissue, decreased expression of the KCC3 protein was observed (\(n = 3\)).

These results suggest that KCC3 is abundantly expressed in the gastric parietal cells and it may be involved in the mechanism of gastric acid secretion.


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