Altered CLC-5 expression in mouse inner medullary collecting duct cells disrupts endocytosis and promotes calcium oxalate crystal adhesion

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Dent's disease, characterised by proteinuria, hypercalciuria, and renal stone formation, results from mutation of the Cl^- channel CLC-5. We have previously demonstrated that mCLC-5 is expressed in inner medullary collecting duct cells (mIMCD-3 cell line) within acidic endosomes (Sayer *et al.* 2001). Here we report the consequence of ablation of mCLC-5 expression upon endocytosis of wheatgerm agglutin (WGA) and upon binding of Ca-oxalate crystals.

mIMCD-3 cells were transiently transfected with expression vector alone (control) (pCDNA3.1/CT-GFP, Invitrogen) or combined with vector containing sense full-length mCLC-5 (with C-terminal stop codon) or antisense mCLC-5, 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 GFP fluorescence and analysed 24–48 h post-transfection. Binding and endocytosis of TRITC-wheatgerm agglutinin (WGA) was followed over a 1 h time course in phosphate-buffered saline (PBS). Optical sections of positive transfectants were collected to allow identification of membrane-bound and internalised WGA. In 3–5 separate experiments, after 1 h, internalisation of WGA occurred in 17/21 of control transfectants and 13/17 of sense mCLC-5 transfectants. With antisense mCLC-5 transfectants endocytosis was disrupted with the majority of cells showing only membrane-bound WGA; only 1/13 of cells showed WGA internalisation ($P < 0.001$ antisense *vs.* combined control/sense, Fisher's exact test).

Calcium oxalate monohydrate crystals were grown in a high calcium (50 mM $CaCl_2$) medium exposed to diethyloxalate vapour. Crystals were harvested and overlaid onto mIMCD-3 cultures for 30 min prior to a brief wash in PBS and CLSM imaging of unfixed cells. In 4–5 separate experiments, in the majority of control or sense transfectants (34/44 and 38/44 cells, respectively), no crystal adhesion was observed. The remaining cells (5/44 or 3/44) showed adhesion of single crystals (< 10 μM size) or agglomerates (5/44 and 3/44 >10 μM size). For antisense mCLC-5 transfectants the majority of cells were associated (33/50, $P < 0.001$ *vs.* control/sense cells, χ^2 test) with crystal agglomerates, whilst 1/50 showed adhesion of single crystals and 16/50 showed no crystal adhesion.

Transfection of antisense mCLC-5 is therefore associated with disruption of endocytosis and aggregation of agglomerates of Ca-oxalate crystals on inner medullary collecting duct cells. Crystal retention and agglomeration at the point of maximal urinary concentration are likely to be key factors in renal stone formation.

Sayer, J.A. *et al.* (2001). *J. Physiol.* **536**, 769–783.

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How do CFTR blockers inhibit the growth of MDCK cysts?

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Polycystic kidney disease is characterised by massive enlargement of fluid-filled epithelial cysts that involves cAMP-dependent cell proliferation and fluid secretion driven by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Sullivan *et al.* 1998). Using MDCK cysts, we previously demonstrated that CFTR blockers inhibit cyst growth (Findlay *et al.* 2001). In this study, we investigated the effects of CFTR blockers on cell proliferation and cAMP-stimulated Cl^- currents to understand how drugs inhibit the growth of MDCK cysts.

We grew cysts as described previously (Findlay *et al.* 2001) with the exception that the effects of drugs were tested between days 6 and 12. For cell proliferation assays, MDCK cells were grown in media containing 0.01 % FBS and cAMP agonists in the absence and presence of drugs over a 6 day period and cell numbers determined using a haemocytometer. Using MDCK epithelia and the Ussing chamber technique, we quantified the effects of drugs on cAMP-stimulated Cl^- currents. We tested the effects of four drugs that inhibit CFTR including the open-channel blockers glibenclamide (100 μM) and NPPB (50 μM) and the allosteric blocker genistein (100 μM). As controls, we used first, tamoxifen (10 μM), calix[4]arene (10 μM) and DIDS (200 μM), which inhibit other Cl^- channels but not CFTR, and second, TEA (10 mM), bumetanide (100 μM) and ouabain (1 μM), which block basolateral membrane channels and transporters in MDCK cells.

There were three sets of results in this study. First, CFTR inhibitors, bumetanide and ouabain reduced dramatically the volume of cysts ($n = 20$–36; $P < 0.05$; one-way ANOVA), whereas TEA, calix[4]arene and DIDS were without effect on cyst growth ($n = 31$–39; $P > 0.05$). Second, with the exception of bumetanide, all the drugs tested inhibited potently cell proliferation ($n = 4$–10; $P < 0.0.1$). Third, CFTR inhibitors, TEA, bumetanide and ouabain all inhibited cAMP-stimulated Cl^- currents ($n = 5$–7; $P < 0.01$; Student's paired t test), whereas calix[4]arene, DIDS and tamoxifen were without effect. To analyse further these data, we compared the magnitude of inhibition of cyst growth, cell proliferation and cAMP-stimulated Cl^- currents by different drugs. Inhibition of cyst growth and cAMP-stimulated Cl^- currents were well correlated (correlation coefficient = 0.71; $P < 0.01$; Pearson's correlation test), whereas inhibition of cyst growth and cell proliferation was not well correlated (correlation coefficient = 0.40; $P > 0.05$; Pearson's correlation test). We interpret these data to suggest that CFTR blockers probably inhibit cyst growth by preventing fluid accumulation within the cyst lumen.

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Effects of hypothermia on renal function in normothermic and cold-acclimated anaesthetised rats

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Acute hypothermia causes a reduction in renal blood flow (RBF), glomerular filtration rate (GFR), natriuresis and diuresis, even though mean arterial blood pressure (MABP) is largely unchanged (Sabharwal *et al.* 2001). This implies an increase in renal vascular resistance (RVR) occurring primarily at the afferent arteriole, and a possible rise in the neurally mediated vascular tone (Broman *et al.* 1995). This study aimed to correlate the changes in the pattern of renal sympathetic nerve activity (RSNA) to cardiovascular and renal functions cooling to 25 °C and on rewarming back to 37 °C, and to compare the response in normothermic and cold-acclimated rats.

Male Wistar rats, 290–320 g ($n = 14$ normothermic, $n = 12$ cold-acclimated), were anaesthetised with fluothane (2.5 % in O_2) and maintained with α-chloralose/urethane (32/450 mg kg^{-1}) via a femoral vein cannula. MABP and heart rate (HR) were measured via a femoral artery cannula interfaced with a digital recording device. An infusion of 1.5 % inulin in saline (150 mM NaCl at 3 ml h^{-1}) was used for the estimation of glomerular filtration rate (GFR), while urine flow (UV) and absolute sodium excretion (UNaV) were determined from the outflow of the left ureteral cannula. For RSNA experiments, a renal nerve bundle of the left kidney was isolated, placed on bipolar electrodes and the signal subjected to power spectral analysis. Core (deep oesophageal) temperature (T_b) was regulated by means of a thermostatted plate. At the end of experiments, rats were killed with an overdose of sodium pentabarbitone. Cold-acclimated rats were exposed to progressively lower environmental temperature (20 °C to 4 °C) and photoperiod (12 h to 1 h light day^{-1}) over 8 weeks. Data (means ± S.E.M.) were analysed using ANOVA and significance taken at $P < 0.05$.

At $T_b = 25$ °C, there was a ~50 % reduction in HR (418 ± 9 b.p.m. at 37 °C, $P < 0.01$) but only a ~15 % fall in MABP (111 ± 2 mmHg at 37 °C, $P < 0.05$). On cooling GFR decreased by ~50 % (4.6 ± 1 ml kg^{-1} min^{-1} at 37 °C, $P < 0.05$) in normothermic and by 5 % (3.8 ± 1 ml kg^{-1} min^{-1} at 37 °C) in cold-acclimated rats. A cold-induced diuresis (40 ± 5 *vs.* 21 ± 2 μl kg^{-1} min^{-1}, $P < 0.05$, in normothermic and 47 ± 10 *vs.* 38 ± 4 μl kg^{-1} min^{-1} in cold-acclimated at 37 °C) and natriuresis (5 ± 1 *vs.* 1 ± 1 μmol kg^{-1} min^{-1}, $P < 0.05$, in normothermic and 5 ± 1 *vs.* 3 ± 1 μmol kg^{-1} min^{-1}, $P < 0.05$, in cold-acclimated at 37 °C) was evident at $T_b = 25$ °C. There was a loss in pulsatility in the RSNA signal while integrated RSNA increased by 20 % ($P < 0.05$) in normothermic but decreased by 20 % ($P < 0.05$) in cold-acclimated rats at $T_b = 25$ °C. There was a progressive fall in proportion of RSNA power at HR frequency with cooling (20 % in normothermic and 80 % in cold-acclimated rats, $P < 0.05$). On rewarming all variables, in both groups of rats, returned to basal levels. We conclude that the natriuresis and diuresis in normothermic rats during hypothermia is a consequence of a reduction in nephron reabsorption and possibly due to the altered patterning of RSNA. In cold-acclimated rats it may be due to altered renal haemodynamics and/or hormonal influences induced by chronic cold exposure.

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All procedures accord with current UK legislation.

Regulation of intracellular [Ca^{2+}] in equine chondrocytes

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Articular chondrocytes survive in an extracellular matrix rich in negatively charged proteoglycans, subject to substantial mechanical stress. This environment presents the challenge of high, fluctuating levels of hydrostatic pressure, osmolality, and [H^+], and relative hypoxia (Wilkins *et al.* 2000). During galloping or jumping, the equine metacarpophalangeal (or fetlock) joint must withstand particularly high pressures. We report the first experiments to investigate the effect of some of these physicochemical variables on free [Ca^{2+}]$_i$ in chondrocytes from this joint.

Articular chondrocytes were isolated by collagenase digestion of cartilage slices taken aseptically from the equine fetlock joint (animal was killed humanely for other purposes, by barbiturate overdose). Cells were loaded with the Ca^{2+}-sensitive fluorophore fura-2 (fura-2 AM, 5 μM). [Ca^{2+}]$_i$ was measured in cell suspensions incubated in a thermostatically regulated (37 °C) cuvette fluorimeter equipped with a magnetic stirrer (Ex. 340 nm/380 nm, Em. 510 nm; Browning & Wilkins, 2002). Following hypotonic shock (osmolality reduced from 290 to 145 mosmol kg^{-1}), [Ca^{2+}]$_i$ increased by 216 ± 66 nM (Fig. 1, trace a). The rise in free [Ca^{2+}]$_i$ was: (i) dependent on hypotonicity, trace b; (ii) greater when free [Ca^{2+}]$_o$ was increased from 2 to 5 mM, trace c; (iii) inhibited in Ca^{2+}-free solutions (nominally 0 [Ca^{2+}]$_i$ plus 100 μM EGTA), trace d; and (iv) retarded by gadolinium (10 μM, an inhibitor of stretch-activated channels), trace e. In addition, thapsigargin (intracellular store Ca^{2+} pump inhibitor) and rises in pH_o both elevated free [Ca^{2+}]$_i$.

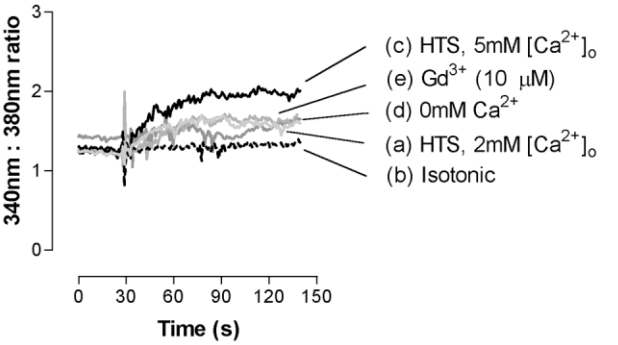

Figure 1. Effect of hypotonic shock on free [Ca^{2+}]$_i$ in equine chondrocytes. Free [Ca^{2+}]$_i$ was determined spectrophotometrically with fura-2 emission. See text for experimental conditions.

These findings indicate that free [Ca^{2+}]$_i$ in equine chondrocytes responds to changes in osmolality, pH and [Ca^{2+}]$_o$, in a similar way to that observed previously in human chondrocytes (Browning & Wilkins, 2002). The response appears to require entry through channels, although intracellular stores are also present and provide an additional source of Ca^{2+}. Free [Ca^{2+}]$_i$ in chondrocytes is an important parameter, a change in whose magnitude affects many cell parameters including matrix synthesis. The equine preparation described here represents a valuable one in which to study further the mechanisms responsible.

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Noradrenaline reverses the SH-NaCl hydro-osmotic response in *Rana temporaria* urinary bladder

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Hypertonic serosal medium (SH) invokes a hydro-osmotic response in anuran urinary bladder similar to that induced by antidiuretic hormone (ADH) (Bentley, 1964). We have reported that the hydro-osmotic response of bladders to medium made hypertonic by addition of 100 mosmol NaCl (SH-NaCl) is reversed by noradrenaline (NA) (Hanna-Mitchell & Gebruers, 2001). Ripoche *et al.* (1973) reported that NA failed to reverse the hydro-osmotic response to medium made hypertonic by addition of mannitol (220 mosmol). This was interpreted as indicating that the SH-hydro-osmotic response did not depend on cAMP for its maintenance, in contrast to ADH-induced water flow.

In this study, we examined the mode of reversal by noradrenaline (50 μM) of SH-NaCl-induced water permeability increases. *Rana temporaria* urinary bladders from humanely killed males were used in gravimetric experiments, employing a modification of the Bentley method. Bath addition of NA was 15 min following imposition of SH-NaCl. Flux (J_w) is expressed as mean cumulative fluid loss (μl 20 min^{-1}), commencing 5 min post-addition of agents to the bath. Statistical analysis employed Student's unpaired *t* test and results are expressed as means ± S.E.M.

Noradrenaline failed to reverse the SH–water response in the presence of 100 μM yohimbine (a specific α_2 -antagonist). Neither 100 μM prazosin (a specific α_1-antagonist) nor 100 μM propranolol (a non-specific β-antagonist) inhibited NA (Table 1).

Table 1. Mean J_w (μl 20 min^{-1}) in bladders exposed to NA in the presence and absence of adrenergic receptor antagonist

	Mean J_w (μl 20 min^{-1})	
SH-NaCl (control)	334.5 ± 69.4	$n = 5$
SH+NA	88.7 ± 23.6	$n = 5$, $P < 0.005$
SH+Yohimbine+NA	242.7 ± 88.6	$n = 5$, $P > 0.05$
SH+Prazosin+NA	71.9 ± 32.9	$n = 6$, $P < 0.005$
SH+Propranolol+NA	142.0 ± 32.0	$n = 4$, $P < 0.005$

P values are statistical comparisons with control.

To exclude a role for prostaglandins, bladders were incubated in indomethacin (10^{-5} M) for 2 h before exposure to SH. The hydro-osmotic response was still reversed by NA (244.2 ± 40.8 μl 15 min^{-1} ($n = 5$) compared with 521.0 ± 89.5 μl 15 min^{-1} ($n = 5$) in control bladders, $P < 0.005$).

Successful RT-PCR using RNA extracted from isolated urinary bladder epithelium, resulted in a product of approximately 520 bp, suggesting that the frog α_2-receptor gene (Hunter & Elgar, 2001) is expressed in *Rana temporaria* bladder.

These results indicate that similar to the ADH-hydro-osmotic response, the SH-NaCl-water response is dependent on cAMP for its maintenance.

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Sheep choroid plexus cells in culture: expression of epithelial phenotype, barrier properties and apical secretion of the CSF

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The aim of this study was to develop and evaluate a primary culture of choroid plexus (CP) epithelial cells as an *in vitro* model for studying transport processes between the plasma and the CSF. Epithelial cells were disseminated from sheep (Clun Forest) CPs by enzymatic digestion. The animals were anaesthetised with thiopenthone-Na^+ (20–25 mg kg^{-1} I.V.), killed humanely and then fourth ventricle and lateral ventricle CPs removed and kept in warm (37 °C) CO_2-independent medium. The CPs were washed and incubated with DMEM (w/o fetal calf serum (FCS), Ca^{2+}, Mg^{2+}) containing proteolytic enzymes and released cells were finally resuspended in DMEM/F12 medium supplemented with FCS, antibiotic/antimicotic solution and hormones and 2×10^5 cells cm^{-2} seeded on polyester inserts (0.4 μm pore) which were uncoated or coated with different basal lamina components. The cells were left in the incubator at 37 °C and 5 % CO_2 for 4–72 h for the attachment to the surface, then the medium was changed first time and subsequently each 2–3 days. The results showed that the digestion of tissue with 0.25 % trypsin (30 min) yielded the maximal number of the cells ($3.4 \pm 0.9 \times 10^6$ cells (100 mg)$^{-1}$, mean ± S.D.) but < 5 % plating efficiency (PE), while mild and short digestion with pronase and trypsin released less cells but the PE was 12.2 ± 4.3 % (mean ± S.D.) (although no difference in the viability of the cells was observed). Cellular attachment and formation of the monolayer depended also on the coating of inserts. Although the lowest PE and longest time for the initial attachment (more than 24 h) was observed on laminin-coated inserts, the cells spread subsequently more rapidly on laminin, with the population doubling time 3–4 days and they made optical confluence at day 4 after seeding showing typical cobblestone-like arrangement. The plated cells maintained epithelial-like morphology and showed positive staining with a mixture of anti-cytokeratin antibodies. The electrical resistance across the monolayer increased with time and reached 87 ± 6Ω cm^{-2} (mean ± S.D.) at day 8, after which no further increase was observed, while the permeability for ^{14}C sucrose and ^{3}H mannitol decreased from 15.0 ± 2.5 and 15.9 ± 3.0 to 3.5 ± 1.0 and $4.9 \pm 1.4 \times 10^{-4}$ cm min^{-1} (means ± S.E.M.), respectively, indicating that the tight junctions were developed, which was also proved by positive staining with anti-occludin antibodies. These cells seem to be highly differentiated since the immunocytochemical study revealed strong positive stain of transthyretin in the cytoplasma (mostly around the nucleus). Another sign of differentiation of these cells was the CSF secretion from the apical side that was detected by measuring the dilution of ^{125}I-albumin or blue dextran over 18 h incubation. When natural sheep CSF was present in the apical (CSF) compartment 12 h before and during the incubation CSF

secretion was 2.32 ± 0.98 μl cm^{-2} h^{-1} (^{125}I-albumin, $n = 4$) and 1.46 ± 0.66 μl cm^{-2} h^{-1} (blue dextran, $n = 5$) (means ± S.E.M.), respectively, and the presence of medium with serum in the apical chamber affected that secretion. These results suggest that this primary cell culture system possesses typical choroidal epithelial characteristics and appears to be a suitable model for *in vitro* studies.

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All procedures accord with current UK legislation.

Signalling pathways involved in rapid non-genomic effects of 17β-oestradiol on the Na$^+$–H$^+$ exchanger in female rat distal colon

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Rapid activation of the Na$^+$–H$^+$ exchanger, in response to steroid hormones, has been demonstrated in a wide variety of tissues including epithelia, vascular smooth muscle and lymphocytes. Non-genomic stimulation of the Na$^+$–H$^+$ exchanger can influence the activity of other ionic transporters, which in turn determine cell volume, secretion and absorption. Previous studies from our laboratory have shown that 17β-oestradiol (E2) activates the Na$^+$–H$^+$ exchanger in female rat distal colon.

Here, we investigated the signalling pathways involved in rapid E2 effects on the Na$^+$–H$^+$ exchanger. The acute response of the Na$^+$–H$^+$ exchanger to E2 was measured on pH$_i$ recovery rate following an ammonium chloride acid load in female Sprague-Dawley rats (killed by cervical dislocation). The distal colon was removed, crypts were isolated, loaded with the pH-sensitive fluorescent dye, 2′,7′-bis(carboxyethyl)carboxyfluorescein (BCECF) and finally pH$_i$ was recorded in the absence of bicarbonate. Data represent means ± S.E.M. of six independent experiments.

We have previously shown (unpublished data) that E2 induces a rapid (< 10 min), non-concentration-dependent activation of the pH$_i$ recovery rate basal = 0.46 ± 0.05 ΔpH units min^{-1}; E2 (10 nM) = 0.96 ± 0.04 ΔpH units min^{-1} ($P < 0.05$, ANOVA test). The rapidity of the E2 action and its insensitivity to the classical steroid receptor antagonist, ICI 182,780, indicate a non-genomic action of E2 on the Na$^+$–H$^+$ exchanger. Evidence from other studies supports the involvement of a membrane receptor in non-genomic responses. To test the involvement of a membrane receptor in our system, we used an impeded form of E2 linked to BSA (E2-CMO-BSA). Both E2 (10 nM) and E2-CMO-BSA (10 nM) induced the same activation of the pH$_i$ recovery rate (E2 (10 nM) = 0.96 ± 0.04 ΔpH units min^{-1}, E2-CMO-BSA (10 nM) = 0.89 ± 0.06 ΔpH units min^{-1}; $P > 0.05$). These results are consistent with the presence of a membrane receptor. We subsequently investigated G protein-coupled receptor involvement in our rapid E2 response. The involvement of two G protein subtypes, $G_{\alpha i}$ and $G_{\alpha s}$, were assessed using pertussis toxin (100 ng ml^{-1}) and cholera toxin (100 ng ml^{-1}), respectively. Inhibition of $G_{\alpha s}$ led to a significant reduction of the E2-induced pH$_i$ recovery rate (E2 = 0.98 ΔpH units min^{-1}, E2 + cholera toxin = 0.60 ΔpH units min^{-1}; $P < 0.05$). Inhibition of $G_{\alpha i}$ was without effect. Phospholipase C (PLC) involvement in E2 action was assessed using the specific inhibitor, U73122 (800 nM). Pre-incubation with U73122 significantly reduced the E2-induced activation of pH$_i$ recovery rate by 30 %. The role of intracellular calcium was demonstrated using the cell-permeant calcium chelator, BAPTA AM (50 mM). Pretreatment with BAPTA AM inhibited the E2-induced activation of pH$_i$ recovery rate by 40 % (E2 = 0.98 ± 0.04 ΔpH units min^{-1}, E2 + BAPTA AM = 0.59 ± 0.04 ΔpH units min^{-1}; $P < 0.05$).

This study demonstrates a rapid non-genomic activation of the Na$^+$–H$^+$ exchanger in isolated female rat distal colonic crypts by E2. This early response to E2 appears to involve a cholera toxin-sensitive G protein-coupled membrane receptor, the identity of which is unknown. Phospholipase C and intracellular calcium are also involved in the rapid E2 effect on the Na$^+$–H$^+$ exchanger. This early response to E2 may result in an increase in the NaCl absorptive capacity of the colon and/or modulation of pH$_i$-sensitive ion channels.

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All procedures accord with current National guidelines.

Activity of Na$^+$–H$^+$ exchanger isoforms in the syncytiotrophoblast of the term human placenta

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Western blotting data suggest that the Na$^+$–H$^+$ exchanger (NHE) isoforms, NHE1, NHE2 and NHE3, are expressed on the maternal-facing microvillous plasma membrane (MVM) of the human placental syncytiotrophoblast (Hughes *et al.* 2000; Pepe *et al.* 2001). NHE1 and NHE 3 are expressed on the fetal-facing basal plasma membrane (BM) (Pepe *et al.* 2001). Here, we used specific inhibitors of NHE1/NHE2 and NHE3 to investigate and compare the functional activity of NHE isoforms in MVM and BM vesicles from the term placental syncytiotrophoblast (TPS).

Initial rate of ^{22}Na uptake into vesicles was measured at room temperature in the presence of an outwardly directed proton gradient, without or with NHE inhibitors and expressed as pmol (mg protein)$^{-1}$ min^{-1}, mean ± S.E.M., n = number of placentas. Inhibitor experiments were performed over 30 s and results expressed as pmol (mg protein)$^{-1}$ (30 s)$^{-1}$ (mean ± S.E.M.). Initial rate in MVM was 289 ± 66 ($n = 5$), a significantly higher rate than BM, 71 ± 21 ($n = 4$, $P < 0.05$, Student's unpaired t test). In MVM vesicles, uptake at 30 s was 336 ± 52 ($n = 6$); amiloride (500 μM), a non-specific inhibitor of NHE transport (Mahnensmith & Aronson, 1985), significantly reduced this to 116 ± 47 ($n = 6$, $P < 0.05$, ANOVA followed by Dunnett's *post-hoc* test). HOE 694 (100 μM inhibits NHE1; Counillon *et al.* 1993), reduced uptake to 51 ± 23 ($n = 5$, $P < 0.01$) with an EC_{50} of 0.12 μM. S3226 at 1 μM (inhibits NHE3; Schwark *et al.* 1998) had no effect (262 ± 54, $n = 5$; n.s.). At higher concentrations, S3226 (inhibits NHE1; Schwark *et al.* 1998) uptake was inhibited with an EC_{50} of 4.04 μM. Control uptake of ^{22}Na into BM vesicles at 30 s was 84 ± 11 ($n = 4$), which was unaffected by inhibitors (amiloride, 63 ± 18, $n = 4$; HOE 694, 63 ± 13, $n = 4$; S3226, 95 ± 12, $n = 4$).

In conclusion, these data suggest that NHE3 is not a functional isoform in MVM vesicles isolated from the TPS. ^{22}Na uptake activity in the BM is 25 % of that seen in the MVM and was insensitive to all inhibitors, suggesting that NHE is not active in this membrane under these conditions. The polarization observed in this study may be of physiological significance with regard to maternofetal Na$^+$ and proton exchange and possibly syncytiotrophoblast pH regulation.

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All procedures accord with current local guidelines.

Sodium and bicarbonate reabsorption in NHE3 null mice: evidence for upregulation of NHE2

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NHE3 null mice are only mildly acidotic, despite a profound defect in sodium bicarbonate reabsorption in the proximal tubule (Schultheis *et al.* 1998). Recent evidence suggests that a significant fraction of bicarbonate reabsorption in the distal convoluted tubule is mediated by NHE2 (Wang *et al.* 2001). To assess the possible role of distal tubule NHE2 in compensating for the proximal defect in NHE3 null mice, we have assessed the impact of HOE694, an inhibitor with affinity for NHE2, on excretion of sodium and bicarbonate in both NHE3 null and wild-type mice.

Mice ($n = 12$ in each group) were anaesthetised (Inactin, 100 mg kg^{-1} I.P.) and infused intravenously with a saline solution containing [^{3}H]inulin for the measurement of glomerular filtration rate (GFR). After 1 h of measurements, mice in each group received either HOE694 (3 mg kg^{-1}; 3 mg kg^{-1} h^{-1}) or vehicle (1 % DMSO) alone, and measurements were performed during the subsequent hour. At the end of the experiment, animals were killed by an overdose of anaesthetic. Data are means ± S.E.M. Comparisons were made by ANOVA.

GFR (0.62 ± 0.07 *vs.* 1.05 ± 0.05 ml min^{-1} 100 g^{-1}; $P < 0.05$) and sodium excretion (0.14 ± 0.02 *vs.* 0.21 ± 0.03 μmol min^{-1}; $P < 0.05$) were lower in null mice than in wild-type. Net acid excretion was also reduced in NHE3 null mice, reflecting a reduction in phosphate excretion (0.6 ± 0.3 *vs.* 73.7 ± 8.4 nmol min^{-1}; $P < 0.05$) and an increase in bicarbonate excretion (15.0 ± 2.2 *vs.* 2.5 ± 0.7 nmol min^{-1}; $P < 0.05$). Ammonium excretion was similar in both groups. In wild-type mice, HOE694 had no effect on electrolyte excretion rate. In contrast, both sodium excretion (0.24 ± 0.05 μmol min^{-1}; $P < 0.05$) and bicarbonate excretion (33.5 ± 4.9 nmol min^{-1}) were higher in NHE3 null mice receiving HOE694 than in time controls. The drug did not affect GFR.

These data indicate that the acidosis observed in NHE3 null mice reflects impaired renal acid-base handling. Our results suggest that increased NHE2 activity contributes to a compensatory increase in renal sodium bicarbonate reabsorption although the mechanism of upregulation remains unclear.

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All procedures accord with current National and local guidelines.

Regulatory volume decrease in α-cells isolated from the rat pancreas involves K^+–Cl^- cotransporters

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Regulatory volume decrease (RVD) in α-cells isolated from rat pancreatic islets was examined using video-imaging methods. Rats were humanely killed by stunning and cervical dislocation. Pancreatic islets were isolated by collagenase digestion, and the islets dispersed into single cells in Ca^{2+}-free medium (Miley *et al.* 1997). α-Cells were selected from the islet cell population on the basis of volume, i.e. < 0.8 pl (see Majid *et al.* 2001).

Cells were bathed in isotonic, Hepes-buffered solutions (302 mosmol (kg H_2O)$^{-1}$). On exposure to hypotonic solutions (197 mosmol (kg H_2O)$^{-1}$ by removal of NaCl), relative cell volume increased to a maximum of 1.30 ± 0.04 (mean ± S.E.M.) in nine α-cells. Cell volume then decreased over the remainder of the 15 min exposure to the hypotonic solution, i.e. they exhibited a RVD. The volume recovery (maximum volume − minimum volume) was 0.21 ± 0.02 in control conditions. The RVD in α-cells was inhibited by 10 mM R-(+)-([2-n-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro1-oxo-1H-inden-5-yl]oxy)acetic acid (DIOA; a K^+–Cl^- cotransporter inhibitor, Shen *et al.* 2000). The volume recovery in the presence of DIOA was 0.12 ± 0.03 ($n = 6$; significantly less than control by Student's t test for unpaired data, $P < 0.05$). The RVD in pancreatic β-cells, which involves K^+ and Cl^- channels, was not affected by 10 μM DIOA ($P > 0.1$). These data suggest that K^+–Cl^- cotransporters contribute to the RVD in pancreatic α-cells.

The relative volume of eight α-cells increased from 0.49 ± 0.08 pl in control conditions, to a maximum of 0.52 ± 0.03 pl ($n = 8$) when they were exposed to isotonic solutions containing 10 μM DIOA for 15 min ($P < 0.05$ by paired t test). Cell volume did not change in time-matched isotonic control experiments ($P > 0.1$). These data suggest that K^+–Cl^- cotransporters may be active in α-cells at normal cell volumes in isotonic solutions.

In conclusion, the data are consistent with the expression of K^+–Cl^- cotransporters in pancreatic α-cells.

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All procedures accord with current UK legislation.

The N-terminal blocked amino acid *N*-acetyl-L-phenylalanine is a non-translocated substrate for the mammalian peptide transporter PepT1 expressed in *Xenopus laevis* oocytes

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The N-blocked amino acid *N*-acetyl-L-phenylalanine (Ac-Phe) has previously been shown to be a competitive inhibitor of the peptide transporter PepT1 with a K_i of 1.8 mM (Guha *et al.* 1999; Meredith *et al.* 2000). However, Ac-Phe would not be predicted to bind in the same configuration as other substrates by our current template model of the PepT1 substrate-binding site

(Bailey *et al.* 2000) and therefore was not expected to be a substrate. To test this we have used a trans-stimulation of efflux assay to ascertain whether Ac-Phe is a translocated substrate.

PepT1-expressing *Xenopus laevis* oocytes were injected with 4.6 nl ^{3}H-D-Phe-L-Gln (56 MBq ml^{-1}). Efflux studies were performed by placing five oocytes into 100 μl of medium (95 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 20 mM Tris/Mes; pH 5.5) plus the appropriate test compound (at the K_i or K_m concentration value) for 90 min. After this time, an aliquot of the incubation medium was taken and the oocytes were washed in ice-cold medium and lysed with 2% (w/v) SDS. The oocytes and the aliquot of medium were scintillation counted. All data are means ± S.E.M., with $n = 5$ oocytes per data point.

As can be seen in Fig. 1, the control compound Gly-L-Gln trans-stimulated the efflux of D-Phe-L-Gln with 78.6 ± 3.1% of the peptide remaining in the oocyte after 90 min, whereas for Ac-Phe there was no trans-stimulation (109.6 ± 11.7% remaining). In support of this the amount of radiolabelled peptide in the incubation medium was increased 7.8- and 1.2-fold, respectively. In comparison, the C-terminal blocked dipeptide L-phenyl-alanyl-L-tyrosine-amide (Phe-Tyr-NH_2) stimulated a similar level of trans-stimulation to Gly-L-Gln, consistent with electrophysiological evidence for its translocation into PepT1-expressing oocytes (Beattie & Boyd, 2000). No efflux was seen in non-injected oocytes under the same conditions.

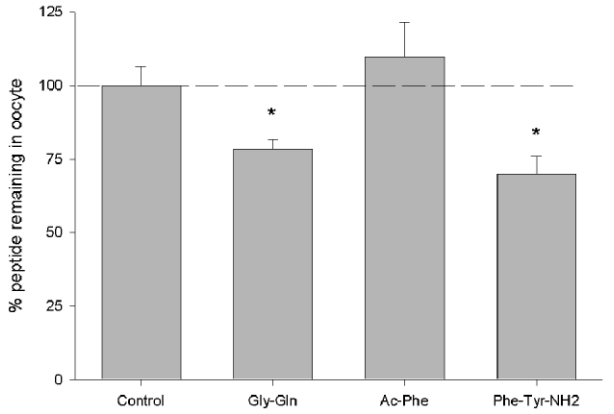

Figure 1. Percentage of injected ^{3}H-D-Phe-L-Gln remaining in the oocytes after 90 min in either incubation medium (control) or medium containing Gly-L-Gln (1.1 mM), Ac-Phe (1.8 mM) or Phe-Tyr-NH_2 (0.95 mM). *$P < 0.05$, Student's unpaired t test.

These data, when taken with the previously available evidence, are consistent with Ac-Phe being a non-translocated competitive inhibitor for PepT1. This finding validates the prediction of the template binding model that Ac-Phe is binding in an atypical manner, and hence should not be translocated.

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We thank M.A. Hediger for the PepT1 clone and The Wellcome Trust for their generous support.

All procedures accord with current UK legislation.

Cell-specific manipulation of second messengers in precisely defined cells by targeted ectopic expression of transgenic receptors

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The combination of physiology and genetics affords opportunities to perform experiments with greater precision than heretofore possible (Dow & Davies, 2001). We illustrate this by selective manipulation of the second messengers cyclic AMP (cAMP), cyclic GMP (cGMP) and calcium (Ca^{2+}) in the renal (Malpighian) tubule of the genetic model organism, *Drosophila melanogaster* (Dow & Davies, 2001). Pharmacological intervention typically involves bathing a whole tissue in a drug, or the use of agents with broad specificities that might confound interpretation. However, ectopic expression of receptors in a cell renders it sensitive to the cognate ligand, and – provided that the necessary internal machinery is present – gives it the potential to respond. In *Drosophila*, it is possible to target such transgenes with great precision, to specific cells in an organotypic context, using GAL4/UAS enhancer traps (Brand & Perrimon, 1993).

Flies were generated that were transgenic for the *Drosophila* 5-HT_7 receptor or the rat atrial natriuretic peptide (rANP) receptor, under control of UAS or heat-shock promoters. Flies transgenic for the *Drosophila* 5-HT_{1A} receptor (Saudou *et al.* 1992) were a gift from L. Maroteaux. Fluid secretion could be stimulated in tubules dissected from such flies by application of 5-HT or rANP, whereas control tubules showed no response. The 5-HT_7 receptor acted to raise cAMP levels, and the rat ANP receptor raised cGMP levels.

UAS-targeted aequorin to measure Ca^{2+} levels in intact tubules showed that 5-HT_{1A} increases Ca^{2+}, with corresponding increases in fluid transport. Furthermore, 5-HT-and rANP-induced signal transduction also increased Ca^{2+} in only principal cells, consistent with the presence of cyclic-nucleotide gated Ca^{2+} channels in this cell-type (MacPherson *et al.* 2001).

The robust tubule transport phenotype makes this system an ideal test-bed for integrative physiology, for example, in investigations of the role of signalling and transport proteins in renal function, and of the *in vivo* function of novel vertebrate receptors. However, this generic technology has potential beyond this specific tissue: in principle, such transgenes can be expressed specifically in any population of cells that can be delineated by a GAL4 driver line.

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All procedures accord with current UK legislation.

Selective blockade of vasopressin V_{1a} receptors does not reduce fractional sodium excretion in anaesthetized rats

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Administration of the V_{1a} receptor antagonist $d(CH_2)_5[Tyr(Me)^2]AVP$ to rats with high vasopressin levels reduces sodium excretion (Musabayane *et al.* 1997; Walter *et al.* 2000), suggesting that stimulation of V_{1a} receptors is natriuretic. However, this antagonist cross-reacts to some extent with oxytocin receptors (Chan *et al.* 2000), and oxytocin is known to increase sodium excretion (Forsling *et al.* 1994; Walter *et al.* 2001). In the present study we have used a recently developed, highly selective V_{1a} receptor antagonist to test the hypothesis that previous findings with the less selective analogue might have resulted from blockade of oxytocin receptors.

Male Sprague-Dawley rats were anaesthetized with Intraval (May & Baker; 100 mg kg^{-1}, I.P.), prepared for clearance studies and laparotomized, as described previously (Walter *et al.* 2001). After a 1 h control period, one group of animals ($n = 10$) received the V_{1a} antagonist $d(CH_2)_5[Tyr(Me)^2, Dab^5]AVP$ (Chan *et al.* 2000; 100 μg bolus, 50 μg h^{-1}; I.V.) for 2 h, while a time-control group ($n = 10$) continued to receive saline alone. This dose of antagonist had previously been shown to block the pressor effect of 10 mU of vasopressin. At the end of each experiment, the rat was killed with an overdose of Intraval. Table 1 shows glomerular filtration rate (GFR), sodium excretion ($U_{Na}V$) and fractional sodium excretion (FE_{Na}) during the control period and during the final hour of antagonist or vehicle infusion (experimental period). The V_{1a} antagonist had no significant effect on these variables; in particular, there was no evidence for a reduction in FE_{Na}.

Table 1. GFR and absolute and fractional sodium excretion (means ± S.E.M.) during the control (C) and experimental (E) periods

		GFR (ml min^{-1})	$U_{Na}V$ (μmol min^{-1})	FE_{Na} (%)
Time controls	C	2.4 ± 0.1	8.3 ± 1.1	2.6 ± 0.4
Time controls	E	2.5 ± 0.1	7.8 ± 0.9	2.3 ± 0.2
V_{1a} antagonist	C	2.4 ± 0.1	8.1 ± 0.9	2.5 ± 0.3
V_{1a} antagonist	E	2.2 ± 0.1	6.5 ± 0.9	2.1 ± 0.3

There were no significant changes in either group of animals (Student's paired *t* test).

These findings with a highly selective V_{1a} antagonist call into question previous claims that V_{1a} receptor stimulation is natriuretic.

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The V_{1a} antagonist was a gift from Professor M. Manning.

All procedures accord with current UK legislation.

Renal calcium homeostasis, calbindin-D_{28K} and plasma calcium ATPase (PMCA) expression in the offspring of diabetic rats

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Offspring from mothers with diabetes mellitus are at risk of altered calcium homeostasis and bone mineral metabolism. Using the streptozotocin rat model of diabetic pregnancy we have previously shown that diabetic mothers show marked hypercalciuria (Birdsey *et al.* 1995). We have also shown that the adult offspring of these rats have, by constrast, reduced urinary calcium output and a remodelling of bone (Hamilton *et al.* 1999). Previous work has also shown altered calbindin-D_{28K} mRNA in kidneys of pregnant diabetic rats and adult offspring from diabetic mothers (Hamilton *et al.* 2000). The aim of this study was to establish whether reduced urinary calcium output was associated with a change in protein expression of calbindin-D_{28K} and PMCA in neonatal offspring of diabetic rats.

Two rat groups were studied: offspring born to control mothers (OC) and offspring born to streptozotocin-induced diabetic mothers (OD). Urine samples were collected following 2 h incubation from neonates and every 24 h over a 3 day period from 8-, 12- and 16-week-old rats. Urinary calcium output was measured using atomic absorption spectrophotometry and expressed as μmol 24 h^{-1} g^{-1} (mean ± S.E.M., n = number of litters); see Table 1. Calbindin-D_{28K} and PMCA protein expression was measured in neonate kidneys using Western blotting and signal was measured in arbitrary density units, normalised to a single standard sample and expressed as a percentage (mean ± S.E.M., n = number of litters).

Table 1.

Treatment	Neonates	8	12	16
OC	0.29 ± 0.04 ($n = 18$)	34.2 ± 4.0 ($n = 6$)	25.0 ± 2.1 ($n = 6$)	26.0 ± 1.0 ($n = 6$)
OD	0.21 ± 0.02 ($n = 12$)	22.1 ± 1.9 ($n = 6$)	15.9 ± 2.2 ($n = 6$)	21.9 ± 0.9 ($n = 6$)

Reduced urinary calcium output was observed in OD when compared with OC in neonates, and 8-, 12- and 16-week rats. Calbindin-D_{28K} (OC = 12.9 ± 2.2 % *vs.* OD = 65.9 ± 11.9 %, $n = 6$, $P < 0.01$, ANOVA) and PMCA (OC = 101.2 ± 4.5 % *vs.* OD = 153.4 ± 8.4 %, $n = 6$, $P < 0.001$, ANOVA) protein expression was significantly higher in neonatal OD than neonatal OC.

In conclusion, these data provide support for previous evidence suggesting that diabetic pregnancy leads to reduced calcium output in offspring and indicate that increased calbindin-D_{28K} and PMCA expression may be partly responsible for this altered renal calcium homeostasis in rats.

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All procedures accord with current UK legislation.

Phloretin-inhibitable urea transport in the mouse colon

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Commensal bacteria that live in the colon express urease and use host-derived urea as a nitrogen source (Fuller & Reeds, 1998). We have previously reported the possible role in this host-microbial relationship of facilitative UT-A urea transporters in the mouse gastrointestinal tract, using an antibody raised against rat UT-A1 (Stewart *et al.* 2001). The aim of this study was to characterise two novel antibodies specifically raised against mouse UT-A proteins, and use them to further investigate the function of UT-A transporters in the mouse colon. Tissue was obtained from humanely killed male adult MF1 mice. Northern blot analysis revealed that four UT-A transcripts were present in mouse colon. These transcripts were different from the known renal mouse UT-A (mUT-A) isoforms, but had similar molecular weights to those in mouse testes. Two antisera, ML446 and ML194, targeted to the N- and C-termini of mUT-A1, respectively, were raised in rabbits. ML446 detected proteins at 34 and 48 kDa in the colon, as well as mUT-A1 (89 and 119 kDa) and mUT-A3 (48–53 kDa) in mouse kidney medulla. ML194 detected proteins at 48, 75 and 100 kDa in both testes and colon, in addition to mUT-A1 and mUT-A2 (43–55 kDa) in kidney medulla. Immunolocalisation in mouse colon using ML446 showed the presence of UT-A proteins in the cytoplasm of cells in the lower third of all colonic crypts. In contrast, ML194 specifically stained the plasma membranes of cells located in the lower portion of colonic crypts, in the proliferative and stem regions that extended to the beginning of the goblet cells. Refractive light flux experiments using colonic plasma membrane vesicles revealed a significant urea flux ($n = 7$, $P < 0.01$, ANOVA). This urea flux was completely inhibited by 500 μM phloretin ($n = 5$, $P < 0.01$, ANOVA), a known inhibitor of facilitative urea transporters.

Our results show that functional UT-A transporters are expressed in the plasma membranes of mouse colonic crypts and are thus ideally situated to transport urea into the colon.

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All procedures accord with current UK legislation.

Acid and base secretion by intact distal airways

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Secretion of HCO_3^- by airway submucosal glands is essential for normal liquid and mucus secretion (Inglis *et al.* 1998). Since the liquid bathing the airway surface is acidic, we have proposed that the surface epithelium may acidify HCO_3^--rich glandular fluid. The aim of this study was to investigate the mechanisms by which intact distal airways modify pH of luminal fluid. Porcine distal bronchi were isolated from humanely killed pigs, cannulated in a bath containing HCO_3^--buffered solution and perfused (3 ml min^{-1}) with similar solution, in which NaCl replaced $NaHCO_3^-$, which was lightly buffered (buffer capacity 0.6 mM pH unit^{-1}) with KH_2PO_4 and NaOH to pH ~7, gassed with 100 % O_2 to eliminate dissolved CO_2 and stirred vigorously. The pH of this circulating luminal solution (10 ml) was monitored continuously. A luminal microelectrode was used to monitor transepithelial potential difference (PD) (Inglis *et al.* 1996). Upon perfusion through the airway lumen pH initially fell by 0.053 ± 0.005 pH units ($[H^+]$ increase 1.56 ± 0.19 μmol h^{-1} (mean ± S.E.M., $n = 22$)) before stabilising (see Fig. 1).

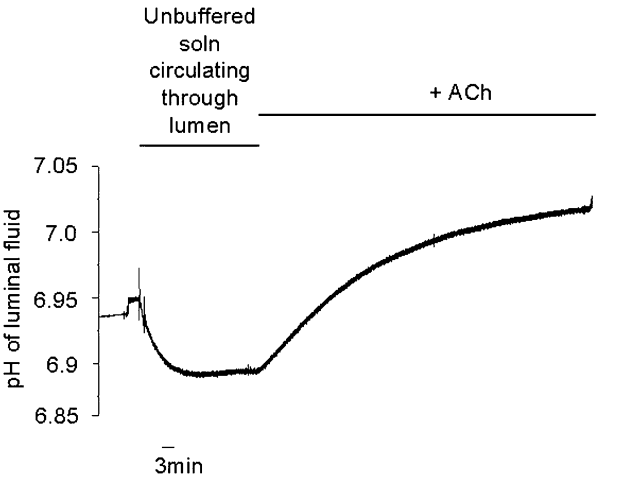

Figure 1. Effect of passing unbuffered solution through airway lumen and addition of ACh to the bathing solution on pH of luminal solution.

Acetylcholine (ACh, 10 μM), a gland secretagogue, increased pH, consistent with HCO_3^- secretion (see Fig. 1). Treatment with DMA (100 μM), a Na^+–H^+ exchanger inhibitor that inhibits gland HCO_3^- secretion (Inglis *et al.* 1998), significantly reduced the rate of alkalinisation induced by ACh (from 0.103 ± 0.023 to 0.028 ± 0.023 pH units h^{-1}, $n = 8$, $P < 0.05$, Student's paired t test used throughout). Removal of HCO_3^- and CO_2 from the bathing solution and treatment with acetazolamide (1 mM) also significantly reduced ACh-induced alkalinisation (0.054 ± 0.006 to 0.0084 ± 0.0008 pH units h^{-1}, $n = 4$, $P < 0.05$). Bumetanide (100 μM), which inhibits Cl^- secretion, significantly increased both resting and ACh-stimulated rates of alkalinisation, consistent with earlier studies suggesting that HCO_3^- secretion is increased after Cl^- secretion is inhibited (Inglis *et al.* 1996). Treatment with NPPB (300 μM) to block the anion channel thought to be required for both Cl^- and HCO_3^- secretion significantly inhibited ACh-induced alkalinisation (0.099 ± 0.024 to 0.023 ± 0.0009, $n = 7$, $P < 0.05$). ACh evoked a depolarisation that was unaffected by DMA, bumetanide or HCO_3^- removal but was abolished by NPPB.

These studies demonstrate that distal airways can both acidify and alkalinise luminal fluid. Stimulation of submucosal glands induces secretion of HCO_3^- by a mechanism that includes generation of intracellular HCO_3^- and concomitant efflux of H^+ by Na^+–H^+ exchange to maintain intracellular pH, and secretion of HCO_3^- through NPPB-sensitive anion channels. Secretion of HCO_3^- is increased following inhibition of Cl^- secretion.

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All procedures accord with current UK legislation.

Multiple P2Y receptors present on apical and basolateral membranes of Calu-3 cells

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Previous studies of the human airway-derived Calu-3 cell line suggested that $P2Y_1$ receptors were the only phospholipase C-coupled P2Y receptor subclass present (Communi *et al.* 1999). However, experiments in which nucleotide-evoked changes in $[Ca^{2+}]_i$ were recorded from cells grown on permeable supports, suggested that at least two P2Y receptor subtypes are present and indicated that these were confined to the basolateral membrane (Clunes *et al.* 2002). We have therefore now explored the effects of apical and basolateral nucleotides upon the short-circuit current generated by Calu-3 cells cultured (10 days) on permeable membranes.

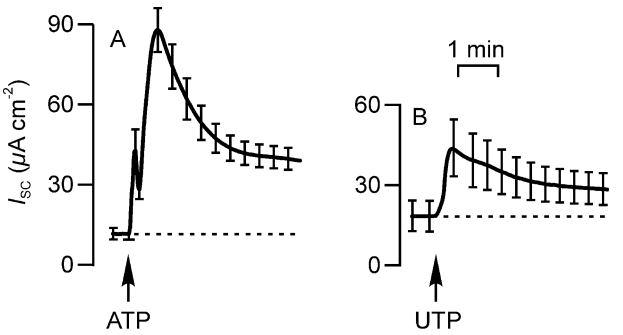

Figure 1. Changes in I_{SC} evoked by adding nucleotides (100 μM) to the solution bathing the basolateral side of Calu-3 cells. The continuous lines shows the mean I_{SC} and vertical bars denote S.E.M.

Basolateral ATP and UTP (100 μM) increased I_{SC} although the integrated response to UTP was only 33.0 ± 14.5 % of that evoked by ATP (Fig. 1). This contrasts with data from the experiments in which $[Ca^{2+}]_i$ was measured, which indicated that basolateral ATP and UTP acted with equal efficacy (Clunes *et al.* 2002). Moreover, the electrometric response to ATP consists of an initial transient followed by a second, slower component, whilst UTP evokes only a monophasic increase (Fig. 1). Experiments in which cells were stimulated with different concentrations of nucleotides showed that the EC_{50} values for ATP and UTP were 48.5 ± 18.1 and 19.8 ± 9.5 μM, respectively. Cross-desensitisation experiments using concentrations of ATP and UTP known to produce essentially complete (> 95 %) autologous desensitisation, showed that ATP-prestimulated cells retained little (22.4 ± 5.5 %, $n = 11$) sensitivity to UTP but that UTP-stimulated cells maintained substantial (59.9 ± 4.0 %, $n = 12$, $P < 0.05$, unpaired t test) sensitivity to ATP. At least two P2Y receptor subtypes are thus present on the basolateral membrane. ATP and UTP (both 100 μM) also increased I_{SC} when added to the apical solution, but the integrated responses were smaller than the response to basolateral ATP (ATP: 33.9 ± 8.4 %, $n = 5$; UTP: 23.8 ± 9.0 %, $n = 5$). Cross-desensitisation experiments indicated that multiple P2Y receptor subtypes were also present in this membrane. Rather than simply expressing $P2Y_1$ receptors (Communi *et al.* 1999), multiple P2Y receptor subtypes seem to be present in both the apical and basolateral membranes of Calu-3 cells, although complementary studies (Clunes *et al.* 2002) suggest that only the basolateral receptors are coupled to changes in $[Ca^{2+}]_i$.

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Increased glomerular angiotensin II binding in the *in utero* protein-restricted rat

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Exposure to a low protein diet during pregnancy results in an increase in offspring blood pressure as early as 4 weeks of age. Previously we have reported that *in utero* protein restriction is associated with a reduction in glomerular number and increased AT_1 receptor expression (Sahajpal & Ashton, 2002). These jointly enhance renal haemodynamic sensitivity in rats exposed to low (9 %) protein, resulting in a significantly greater reduction in GFR than in control (18 %) rats (Sahajpal & Ashton, 2001). We now report differences in glomerular angiotensin II (Ang II) binding in preparations isolated from the renal cortex of rats exposed to 9 and 18 % protein diets.

In utero protein restriction was induced by feeding female Wistar rats a diet containing 9 % protein compared with an isocalorific 18 % protein diet for control animals, from the day of conception until birth. Immediately after birth, dams and pups were fed with a standard maintenance diet. At 4 weeks of age the offspring were humanely killed and kidneys were harvested, decapsulated and chilled in ice-cold phosphate-buffered saline, pH 7.4 (PBS). Cortical tissue was minced, washed through grade sieves with PBS, and centrifuged at 120 *g* at 4°C for 5 min. The pellet was resuspended in PBS, passed under pressure through a 23-gauge needle to remove Bowman's capsule and recentrifuged for a further 5 min. The final yield was > 80 % glomeruli.

Glomerular binding of Ang II was determined by incubating 20 μg of glomerular protein with ^{125}I-labelled Ang II (1×10^{-10} to 1×10^{-7} M) for 45 min in BSA-coated tubes. Non-specific binding was determined by the addition of unlabelled Ang II (5×10^{-5} M) to the incubation medium. After incubation, all tubes were centrifuged at 10 000 *g* for 10 min and the supernatant was aspirated. Each sample was then washed in PBS, recentrifuged and bound activity in the pellet counted in a gamma counter.

Binding of ^{125}I-labelled Ang II by glomeruli from the 9 % protein rats was significantly greater than that by glomeruli from the control 18 % protein rats (18 %, $n = 9$, 159 ± 49 *vs.* 9 %, $n = 7$, 452 ± 75 fmol (mg protein)$^{-1}$, mean ± S.E.M., unpaired t test, $P < 0.01$). The binding affinity constant (K_D) was comparable between both groups (18 %, $n = 9$, 1.82 ± 1.06 *vs.* 9 %, $n = 7$, 0.99 ± 0.26 pmol ^{125}I-labelled Ang II).

These data show that rats exposed to low protein *in utero* have more Ang II binding sites in their glomeruli, although the affinity of these receptors remains unchanged. This supports our previous observation that protein restriction results in a significant increase in AT_1 receptor protein expression (Sahajpal & Ashton, 2002). Increased sensitivity to Ang II coupled with fewer glomeruli may account for the elevated blood pressure observed in these rats.

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All procedures accord with current UK legislation.

Reduction of the expression and function of a green fluorescent protein-tagged calcium-sensing receptor by an antisense cDNA construct

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The extracellular calcium-sensing receptor (CaR) is the first receptor to be identified that has an inorganic cation as its physiological agonist. It was initially isolated from parathyroid glands (Brown *et al.* 1993), where it plays a major role in maintaining normocalcaemia by regulating parathyroid hormone secretion. Subsequent studies have identified the CaR in many other tissues not involved in mineral ion metabolism, where its role is unclear. It was the purpose of this work to develop tools to understand the role of the CaR outside the calcium homeostatic system. Initially, a green fluorescent protein-tagged CaR construct (CaR-GFP) was produced and transiently transfected in an established cell line, human embryonic kidney (HEK)-293 cells. Western blotting and immunofluorescence microscopy performed using anti-GPF peptide and anti-CaR polyclonal antibodies showed that the expression of the GFP-tagged receptor was comparable to that of the non-GFP-tagged CaR. Functional activation of the GFP and non-GFP-tagged receptors was assessed by measuring agonist-induced changes in intracellular calcium concentration ($[Ca^{2+}_i]$) with the calcium-sensitive fluorescent dye fura-2. Our results from three independent experiments show comparable increases in Ca^{2+}_i levels in CaR-GFP and CaR-transfected cells.

We then assessed the ability of a CaR antisense cDNA construct to reduce CaR expression and function in HEK-293 cells. Cells were transiently transfected with CaR-GFP together with: (1) a cDNA construct coding for the antisense sequence of amino acid 1–332 of the rat kidney CaR (CaR-antisense), (2) a cDNA construct coding for the sense sequence of amino acid 1–332 of the rat kidney CaR (CaR-sense), (3) empty vector (pcDNA3.1). 24 h after transfection, confocal microscopy experiments showed a remarkable reduction in the expression of CaR-GFP in cells co-transfected with CaR-antisense cDNA, but not in those co-transfected with the sense-CaR cDNA or with the empty vector. The reduction in CaR-GFP immunoreactivity, measured by Western analysis, amounted to ~70 % in the antisense. Co-transfection of the antisense-CaR cDNA with CaR-GFP in a molar ratio of 1:3 significantly reduced ($n = 3$, $P < 0.05$, paired *t* test) the expected induction of an increase in $[Ca^{2+}_i]$ (fura-2 fluorescence) evoked by CaR agonists.

The present results show that a CaR-antisense cDNA can be used as a tool to modulate the expression and function of CaR in cells expressing the receptor. The availability of GFP-CaR and CaR antisense constructs will allow us to characterise several CaR-mediated processes such as signalling, internalisation and trafficking.

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Gene regulation of renal Ca^{2+} transport proteins by dietary Ca^{2+} in 1α-OHase knock-out mice

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Pseudovitamin D-deficiency rickets (PDDR) is an autosomal disease, characterized by growth retardation, hyperparathyroidism, rickets and undetectable levels of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Mice homozygous for the 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) gene presented the same clinical phenotype as patients with PDDR. To determine whether the severe hypocalcaemia in PDDR is secondary to disturbed $1,25(OH)_2D_3$-stimulated Ca^{2+} reabsorption, the effect of 1α-OHase gene disruption was examined on serum Ca^{2+} and renal expression of the epithelial Ca^{2+} channel (ECaC1), the calbindins, Na^+–Ca^{2+} exchanger (NCX1) and Ca^{2+}-ATPase (PMCA1b) in 1α-OHase knockout mice. Animals were humanely killed according to the guidelines of the Animal Welfare committee. Both serum Ca^{2+} (1α-OHase$^{-/-}$ mice: 1.20 ± 0.05 *versus* 1α-OHase$^{+/-}$ mice: 2.19 ± 0.01 mM) and mRNA/protein expression of ECaC1 (41 ± 3 %, mean ± S.E.M., $n = 6$), calbindin-D_{28K} (31 ± 2 %), calbindin-D_{9K} (58 ± 7 %), NCX1 (10 ± 2 %), PMCA1b (69 ± 3 %) were significantly decreased in 1α-OHase$^{-/-}$ mice. Feeding the 1α-OHase$^{-/-}$ mice a Ca^{2+}-enriched diet normalized expression of these Ca^{2+} proteins except for the calbindin-D9K expression that was not significantly altered. In contrast to 1α-OHase$^{+/-}$ mice in which dietary Ca^{2+} reduced (ECaC1, calbindin-D28K, calbindin-D9K), or did not change (NCX1, PMCA1b) the expression levels of the Ca^{2+} transport proteins. Elevation of the Ca^{2+} transport proteins by dietary Ca^{2+} was accompanied by normalization of the serum Ca^{2+} concentration in 1α-OHase$^{-/-}$ mice from 1.20 ± 0.05 to 2.33 ± 0.10 mM. Like dietary Ca^{2+}, $1,25(OH)_2D_3$ repletion resulted in increased expression of the Ca^{2+} transport proteins and normalization of serum Ca^{2+} concentrations. Immunohistochemistry showed that ECaC1, calbindin-D28K and calbindin-D9K are co-expressed in the majority of the distal tubules of 1α-OHase$^{-/-}$ mice. In addition, 1α-OHase$^{-/-}$ mice, exposed to the high Ca^{2+}-enriched diet, contained immunopositive distal tubules expressing calbindin-D_{28K}, whereas these tubules did not contain calbindin-D_{9K}. Importantly, confocal microscopy demonstrated that the localization of the Ca^{2+} transport proteins is clearly polarized in the renal cell in which ECaC1 is localized along the apical membrane, calbindin-D_{28K} in the cytoplasm and calbindin-D_{9K} along the basolateral membrane resulting in a comprehensive mechanism facilitating renal transcellular Ca^{2+} transport.

All procedures accord with current National and local guidelines.

Immunolocalisation of $P2Y_2$ receptor in human cystic fibrosis eccrine sweat glands.

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The purinoceptor agonist ATP has been shown to increase $[Ca^{2+}]_i$ levels in a sweat gland cell line derived from humans (Wilson *et al.* 1994) and in cells derived from primary sweat gland cultures (Bovell *et al.* 2000). ATP can also induce sweating in isolated glands (Sato *et al.* 1991); however, the mechanism and physiological relevance is not entirely understood. These studies suggest the presence of a P2Y receptor, which we have recently localised in normal human glands (Lindsay *et al.* 2002). Extracellular nucleotides have also been proposed as a means of alleviating some of the symptoms of the autosomal recessive illness, cystic fibrosis (CF), although the localisation of such a purinoceptor in CF sweat glands has never been investigated. Therefore, immunohistochemistry was employed to investigate the localisation of the $P2Y_2$ receptor in human CF eccrine sweat glands and compare this with the expression in normal glands.

Skin biopsies were obtained with informed consent and local medical ethical committee approval from patients suffering from CF ($n = 4$) and from patients with no apparent skin disease ($n = 11$). Samples were fixed, processed and sectioned using standard techniques. Immunohistochemical staining was performed using rabbit antibodies raised against the $P2Y_2$ receptor (Alomone Labs), employing the avidin-biotin complex (ABC) procedure (Vector Labs). Sections were haematoxylin counterstained, dehydrated, cleared, mounted and viewed using light microscopy.

The eccrine reabsorptive duct of CF glands contained dark $P2Y_2$-like immunoreactivity localised to the apical membrane, which was similar to the staining found in normal glands. However, CF glands exhibited staining on the basolateral membranes, which was not seen in normal glands. The secretory coil of CF glands exhibited no staining using both citrate buffer antigen retrieval and no antigen retrieval methods. However, normal glands showed $P2Y_2$-like immunoreactivity localised to the myoepithelial cells of the secretory coil, with some staining seen in the coil itself. Preabsorption of the antibody with the appropriate control peptide abolished all specific staining.

The presence of apical $P2Y_2$-like immunoreactivity in the eccrine sweat gland duct of both normal and CF glands suggests that apical regulation of absorption is important, which has been shown to be the case in colonic epithelia (Cliff & Frizzell, 1990). The reabsorptive duct of CF sweat glands elicited a wider distribution of the $P2Y_2$ receptor compared with normal glands. Those receptors present on the duct may be involved in salt reabsorbtion, as CF glands cannot reabsorb any salt through CFTR. Although the myoepithelial cells of the sweat gland are not regarded as cells involved in either secretion or reabsorption of sweat, the presence of $P2Y_2$ receptor in normal glands would suggest that they do play a role. The absence of any staining in the secretory coil of CF glands may be a result of the condition.

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All procedures accord with current local guidelines.

Nucleotide-evoked Ca^{2+} transients in monolayer cultures of Calu-3 cells reveal multiple P2Y receptor subtypes on the basolateral membrane

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P2Y-mediated Ca^{2+} signals are well documented in cultured epithelial cells but most such studies have been undertaken using cells grown on impermeable supports, which often fail to become polarised. As cell polarity can be an important determinant of P2Y receptor expression (Clunes *et al.* 1998; Wilson *et al.* 1998), we now explore the effects of nucleotides upon intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in Calu-3 cells grown to confluence (3–4 days) on collagen-coated, permeable membranes (Costar Transwell-Col). These cells were loaded with fura-2 by incubation in Hepes-buffered physiological saline containing 3 μM fura-2 AM and 2.5 mM probenecid, and mounted in a chamber that allowed the apical and basolateral sides of the cultured epithelium to be superfused independently (2–3 ml min, 37 °C). Fura-2 fluorescence ratios were recorded using an inverted microscope equipped with extra long working distance optics. All numbers are means ± S.E.M.

Apical ATP (100 μM, $n = 6$) and UTP (300 μM, $n = 6$) failed to evoke discernible responses but were effective basolaterally. Experiments ($n > 4$) in which increasing concentrations of P2Y receptor agonists (0.1–300 μM) were delivered to the basolateral membrane as 30 s pulses revealed a rank order of potency (EC_{50}) of ADP-β-S (8.4 ± 1.1 μM) ≈ ATP (10.7 ± 1.9 μM) > UTP (53.7 ± 6.0 μM). The maximal response to ADP-β-S was only ~50 % of that evoked by ATP, suggesting that this $P2Y_1$ receptor agonist acts as a partial agonist. ATP-stimulated cells (100 μM) became essentially insensitive (6 ± 1 % of control sensitivity, $n = 4$) to UTP, whereas cells exposed to a maximally effective concentration of UTP (300 μM) retained 30 ± 10 % of their sensitivity to ATP. Calu-3 cells thus seem to express a complex population of P2Y receptors in the basolateral, but not the apical membrane. This is surprising as functional studies have shown that apical nucleotides do evoke increased ion transport in these cells (Chambers *et al.* 2002). Moreover our findings also contrast with earlier data from cells grown on impermeable substrates which indicated that UTP-sensitive receptors are not expressed by Calu-3 cells (Communi *et al.* 1999).

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Role of P2X receptors in volume regulation in renal proximal tubule cells isolated from frog

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Extracellular ATP activates P2X purinoceptors, a class of receptors that form Ca^{2+}-permeable channels. It has previously been observed that primary cultures of human renal proximal tubule cells release ATP in response to cell swelling, and that this ATP may then activate P2X receptors leading to a rise in intracellular Ca^{2+} (Wilson *et al.* 1999). A previous study has also reported that single proximal tubule cells isolated from frog undergo an extracellular Ca^{2+}-dependent regulatory volume decrease (RVD) in response to hyposmotic challenge (Robson & Hunter, 1994). The aim of the following study was to investigate whether release of ATP and activation of P2X receptors may play a role in RVD in the renal proximal tubule.

Frogs were killed humanely by cervical dislocation and single proximal tubule cells isolated from the kidneys by enzyme digestion (Hunter, 1989). Cell length was measured using an optical technique (Robson & Hunter, 1994). The bath contained a high Na^+, low K^+ amphibian Ringer solution that contained 89 mM mannitol. This Ringer solution was made hypotonic by the removal of 40 mM mannitol. RVD was examined under the control circumstance and in the presence of (i) 3 units ml^{-1} ADP/ATP apyrase, (ii) 2 mM brilliant blue G (BBG) (a $P2X_7$ antagonist) and (iii) 120 nM KN-62 (a $P2X_7$ antagonist). Data are expressed as means ± S.E.M. Statistical analysis was performed using ANOVAs and significance was assumed at the 5% level.

Under the control circumstance cell length was 21.23 ± 0.34 μm ($n = 40$). This increased by 0.8 ± 0.04 μm on exposure to a hypotonic shock and subsequently decreased by 0.68 ± 0.06 μm on RVD. At steady state the length was 0.13 ± 0.05 μm above the initial control level. In the presence of ATP/ADP apyrase, BBG or KN-62 RVD was inhibited. Steady-state lengths were 0.52 ± 0.12 ($n = 7$), 0.46 ± 0.08 ($n = 9$) and 0.36 ± 0.1 μm ($n = 24$) above the control level for apyrase, BBG and KN-62, respectively.

In summary, these data support the hypothesis that swelling of renal proximal tubule cells leads to the release of ATP and subsequent activation of purinoceptors. The inhibitory effect of BBG and KN-62, both of which are $P2X_7$ antagonists, supports a role for $P2X_7$ in the RVD response. Activation of these P2X receptors may provide the entry pathway for extracellular Ca^{2+} during RVD in the renal proximal tubule.

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All procedures accord with current UK legislation.

Chronic hypoxia upregulates expression of adenosine A_1 receptors in DDT_1 MF-2 cells

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Some forms of acute renal failure (ARF) are associated with upregulation of renal adenosine A_1 receptors (Smith *et al.* 2000) and this may account for the enhanced renal vasoconstrictor response to adenosine noted in ARF induced by myohaemoglobinuria (Gould *et al.* 1995). Since hypoxia/ischaemia are initiating factors for ARF, we have investigated whether chronic hypoxia alters the expression of adenosine A_1 receptor in DDT_1 MF-2 cells, a hamster smooth muscle cell line.

Binding characteristics of [^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine ([^{3}H]DPCPX), a selective adenosine A_1 receptor antagonist, were determined at 37 °C with membranes and whole cells. Binding studies were conducted following 1–16 h culture in either normoxic (P_{O_2} ~142 mmHg) or hypoxic (P_{O_2} ~18 mmHg) conditions. Data are given as means ± S.E.M. ($n = 3$).

Hypoxia of 4–16 h resulted in significant ($P < 0.05$, Student's unpaired t test) increases of up to 60% in the percentage of [^{3}H]DPCPX bound to intact DDT_1 MF-2 cells. Analysis of binding isotherms with membranes isolated from cells exposed to 16 h of hypoxia (Fig. 1) showed a significant increase ($P < 0.05$) in B_{max} (1.7 ± 0.5 *vs.* 0.48 ± 0.02 pmol (mg protein)$^{-1}$) with no detectable change in A_1 receptor affinity as indicated by similar K_d values (1.2 ± 0.3 *vs.* 0.84 ± 0.2 nM). The findings with isolated membranes suggest that the increase in binding noted with intact cells is a result of increased receptor number. Incubation of cells for 4 h with either actinomycin D (5–20 μg ml^{-1}) or cycloheximide (1–10 μM) markedly attenuated the hypoxia-induced increase in percentage binding of [^{3}H]DPCPX. For example, 4 h of hypoxia resulted in an increase in [^{3}H]DPCPX binding of 67 ± 14%, whereas in the presence of 1 μM cycloheximide the increase in binding was only 17 ± 9% ($P < 0.01$).

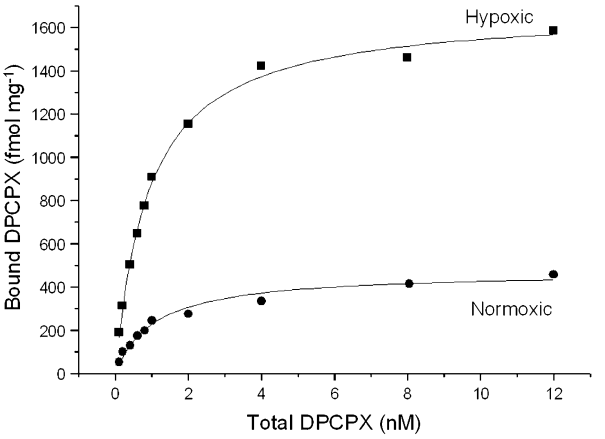

Figure 1. Effect of 16 h of hypoxia on the specific binding of [^{3}H]DPCPX (0.1–12 nM) to membranes from DDT_1 MF-2 cells. Mean data are shown ($n = 3$) and S.E.M. bars have been omitted for clarity.

In summary, this study has shown that chronic hypoxia upregulates adenosine A_1 receptors in smooth muscle cells. This response to hypoxia appears to be a consequence of increased gene transcription and subsequent synthesis of receptor protein. These findings suggest that hypoxia associated with ARF may trigger the upregulation of adenosine A_1 receptors in renal vascular smooth muscle.

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Effect of cross-fostering on blood pressure and renal function in the New Zealand genetically hypertensive rat

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Cross-fostering lowers adult blood pressure in the spontaneously hypertensive rat (SHR) (Gouldsborough & Ashton, 2001) and the Dahl salt-sensitive rat (Murphy & McCarty, 1989). The mechanisms involved are unclear, but involve the kidney and the renin-angiotensin system in the SHR (Gouldsborough & Ashton, 2001). As both of these rat models show salt sensitivity, the aim of this study was to determine whether exposure to a normotensive dam is effective in lowering adult blood pressure in a salt-insensitive model, the New Zealand genetically hypertensive (GH) rat (Ledingham *et al.* 1990).

GH and normotensive (N) rat pups were cross-fostered to a dam of the opposite strain on the day of birth. Systolic blood pressure was measured by tail cuff plethysmography from the age of 6–17 weeks. At 19–20 weeks rats were anaesthetised (thiopentone sodium 100 mg kg^{-1}) and infused with 0.9 % saline and clearance markers (inulin and para-aminohippuric acid, 100 μl min^{-1} I.V.). Following a 3 h equilibration period, urine samples were collected every 20 min and plasma samples (0.5 ml) were collected every hour for a further 3 h. Animals were humanely killed at the end of the experiment.

Cross-fostering GH rats (GHX) significantly reduced their systolic blood pressure compared with naturally reared GH rats up to the age of 9 weeks (GH 192 ± 5 *vs.* GHX 174 ± 3 mmHg, $P < 0.05$, repeated measures ANOVA); thereafter blood pressure did not differ. Cross-fostering N rats (NX) increased their blood pressure compared with naturally reared N rats up to the age of 10 weeks (N 124 ± 3 *vs.* NX 136 ± 3 mmHg, $P < 0.001$); thereafter blood pressure did not differ.

Cross-fostering had little effect on renal function in adult rats, although GHX rats had a significantly lower ($P < 0.05$) glomerular filtration rate (Table 1) and renal blood flow than N rats. Electrolyte handling and fluid output were unaltered in either GHX or NX rats compared with naturally reared rats.

Table 1. Glomerular filtration rate (GFR), urine flow rate (UV) and sodium excretion ($U_{Na}V$) in naturally reared and cross-fostered N and GH rats

	GFR (ml min^{-1})	UV (μl min^{-1})	$U_{Na}V$ (μmol min^{-1})
N ($n = 6$)	1.2 ± 0.2	7.1 ± 2.5	0.31 ± 0.17
NX ($n = 6$)	1.1 ± 0.2	10.1 ± 2.5	0.48 ± 0.15
GH ($n = 5$)	1.0 ± 0.1	7.9 ± 2.2	0.33 ± 0.14
GHX ($n = 5$)	0.5 ± 0.1*	4.8 ± 1.0	0.10 ± 0.05

All values are means ± S.E.M. corrected to 100 g body weight. *$P < 0.05$ *vs.* control N rats, one-way ANOVA.

These data show that cross-fostering is only effective in lowering blood pressure in the young GH rat, in contrast to SHR and Dahl rats in which cross-fostering permanently lowers blood pressure. Interestingly, cross-fostering increased blood pressure in N rats, which is not seen in control WKY and Dahl salt-resistant strains. Renal function appeared to be unaffected in adult rats in which blood pressure had returned to levels comparable with naturally reared animals.

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All procedures accord with current National and local guidelines.

Effect of relaxin on renal electrolyte handling in the anaesthetised rat

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Pregnancy is associated with changes in renal haemodynamics and electrolyte handling. Recent evidence suggests that the ovarian hormone relaxin (RLX) may contribute to these changes. Infusion of RLX for 5 days resulted in a 20–40 % increase in glomerular filtration rate (GFR) and effective renal plasma flow in conscious male rats (Danielson *et al.* 1999). However, this paper did not report the effects of RLX on electrolytes, hence the aim of this study was to determine whether RLX alters renal electrolyte handling.

Under isoflurane anaesthesia, male Sprague-Dawley rats were implanted with an osmotic minipump (Alzet model 2001) loaded with either recombinant human RLX (rhRLX, 4 μg h^{-1}, $n = 9$, a gift from Dr E. Unemori, Connetics Corp., USA) or vehicle (20 mM sodium acetate, $n = 7$). After 7 days animals were prepared for renal function study. Under Intraval anaesthesia (100 mg kg^{-1} sodium thiopentone) rats received euvolaemic fluid replacement (0.9 % saline, [^{3}H]inulin, para-aminohippuric acid) of spontaneous urine output using a servo-controlled system (Burgess *et al.* 1993). After a 3 h equilibration period, urine and plasma samples were taken over a further 3 h. Animals were humanely killed at the end of the experiment.

After 7 days administration of rhRLX effective renal blood flow (ERBF) and urine flow rate were significantly higher than in control rats ($P < 0.05$, ANOVA), but GFR was unaltered. Sodium excretion tended to be higher, while potassium excretion tended to be lower in rhRLX-treated rats, but these differences did not achieve statistical significance. However the Na^+:K^+ ratio was significantly increased ($P < 0.05$). Plasma sodium and potassium concentrations and plasma osmolality were significantly lower ($P < 0.05$) in rhRLX-treated rats (Table 1).

Table 1. Renal function and plasma electrolytes in rats treated with rhRLX

	Control ($n = 7$)	rhRLX ($n = 9$)	P
ERBF (ml min^{-1} 100 g^{-1})	1.2 ± 0.2	2.5 ± 0.3	0.002
GFR (ml min^{-1} 100 g^{-1})	0.57 ± 0.05	0.66 ± 0.05	0.206
UV (μl min^{-1} 100 g^{-1})	3.1 ± 0.3	4.8 ± 0.6	0.031
$U_{Na}V$ (μmol min^{-1} 100 g^{-1})	0.75 ± 0.14	1.23 ± 0.18	0.07
U_KV (μmol min^{-1} 100 g^{-1})	0.93 ± 0.03	0.83 ± 0.04	0.064
Na^+:K^+ ratio	0.8 ± 0.2	1.5 ± 0.3	0.044
P_{Na} (mmol l^{-1})	150.0 ± 1.6	143.5 ± 2.1	0.034
P_K (mmol l^{-1})	3.8 ± 0.1	3.5 ± 0.1	0.038
P_{Osm} (mosmol kg^{-1})	303.6 ± 3.4	293.1 ± 3.2	0.045

Data are shown as means ± S.E.M.

rhRLX induced changes in renal function in male rats which resemble those seen in pregnancy, namely an increase in ERBF and urine output, coupled with a reduction in plasma sodium and osmolality. The lack of apparent effect on GFR may reflect the influence of anaesthesia. These data suggest that RLX may affect renal electrolyte handling in addition to its previously reported effects on haemodynamics.

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All procedures accord with current UK legislation.

Renal action of acute chloroquine and paracetamol administration in the anaesthetised, fluid-balanced rat

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Chloroquine, widely used in the treatment of malaria and rheumatoid arthritis, increases renal nitric oxide synthesis (Ahmed *et al.* 1999), which appears to mediate its diuretic action (Ahmed *et al.* 2000). We have also reported that acute chloroquine administration in rats treated with paracetamol for 30 days induced an antidiuresis which was associated with an increased plasma vasopressin concentration (Ahmed *et al.* 2001). In this study we examined the effect of acute co-administration of chloroquine and paracetamol on renal function.

Sprague-Dawley rats ($n = 6$ per group) were anaesthetised (Intraval 110 mg kg^{-1} I.P.) and prepared for I.V. infusion of dextrose (2.5 %). Following 4 h equilibration, animals received either vehicle, chloroquine (Chq, 0.04 mg h^{-1}), paracetamol (Para, loading dose of 210 mg kg^{-1} followed by 110 mg kg^{-1} for 2 h) or combined chloroquine and paracetamol. Animals were humanely killed at the end of the experiment.

Chloroquine infusion alone was associated with a significant increase in urine flow rate (UFR) (Chq 5.6 ± 0.4 *vs.* vehicle 3.3 ± 0.2 ml h^{-1}, means ± S.E.M., ANOVA, $P < 0.05$), glomerular filtration rate (GFR) (Chq 4.2 ± 0.5 *vs.* vehicle 2.5 ± 0.3 ml min^{-1}, $P < 0.05$) and sodium excretion (Chq 124 ± 22 *vs.* vehicle 36 ± 2 μmol h^{-1}, $P < 0.05$) while paracetamol alone reduced UFR (Para 2.0 ± 0.2 *vs.* vehicle 3.3 ± 0.2 ml h^{-1}, $P < 0.05$), GFR (Para 1.7 ± 0.3 *vs.* vehicle 2.5 ± 0.3 ml min^{-1}, $P < 0.05$) and sodium excretion (Para 12 ± 2 *vs.* vehicle 36 ± 2 μmol h^{-1}, $P < 0.05$). Co-administration of paracetamol with chloroquine blocked the increases in UFR (Chq/Para 2.1 ± 0.2 *vs.* Chq 5.6 ± 0.4 ml h^{-1}, $P < 0.05$), GFR (Para/Chq 2.9 ± 0.2 *vs.* Chq 4.2 ± 0.5 ml min^{-1}, $P < 0.05$) and sodium excretion (Para/Chq 20 ± 5 *vs.* Chq 124 ± 22 μmol h^{-1}, $P < 0.05$) observed with chloroquine alone.

Acute administration of paracetamol appeared to block the renal actions of chloroquine. These data conflict with our earlier observations in rats treated with paracetamol for 30 days prior to chloroquine infusion. In these animals UFR fell, GFR remained the same and sodium excretion increased. This suggests that paracetamol may modify the renal action of chloroquine by different mechanisms depending on the prior period of exposure to paracetamol.

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All procedures accord with current UK legislation.

Hypoxic activation of nitric oxide synthase is p44/42 MAPK dependent and is attenuated by dexamethasone in H441 cells and rat pseudoglandular lung explants

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Several studies suggest that low ambient P_{O_2} is a factor that limits epithelial Na^+ transport (e.g. Ramminger *et al.* 2000), but the mechanism remains unclear. Nitric oxide-evoked inhibition of epithelial Na^+ transport contributes to pulmonary oedema in lung injury (Pittet *et al.* 2002), and by implication, may repress Na^+ transport in the fetal lung. We therefore sought to determine whether fetal pseudoglandular lung tissue exhibits a suitable distribution and activity of nitric oxide synthase (NOS) isoforms to effect repression of epithelial Na^+ transport.

Lung sections from day 16 (G16) rat fetuses were immunostained using antibodies against inducible (i) or endothelial (e) NOS. Both isoforms localised to airway structures with iNOS confined to the distal columnar epithelium and eNOS in the basement membrane region of the proximal airways. Total NOS enzyme activity in G16 and adult lung was 0.17 ± 0.07 (mean ± S.E.M.; $n = 4$) and 0.71 ± 0.21 fmol citrulline (mg protein)$^{-1}$ min^{-1} (fmol mg^{-1} min^{-1}), respectively. To determine the effect of P_{O_2} on NOS activity, G16 lung explants were cultured at either fetal (23 mmHg) or ambient (142 mmHg) P_{O_2} for 24 h with or without 50 μg ml^{-1} *E. coli* lipopolysaccharide (LPS). Basal NOS activity at fetal P_{O_2} was 1.6 ± 0.95 fmol mg^{-1} min^{-1}, rising 2.3-fold with LPS. Basal NOS activity was markedly lower at ambient P_{O_2} (0.49 ± 0.33 fmol mg^{-1} min^{-1}) and was not raised by LPS. Epithelial NO production (as nitrite) was determined using the Griess assay in a human (H441) lung epithelial cell line maintained in RPMI medium containing 10 % dialysed fetal calf serum. At fetal P_{O_2}, basal nitrite production was 21.6 ± 0.9 μM and was 1.5-fold greater than that at ambient P_{O_2} ($P < 0.05$; ANOVA *post-hoc* Tukey's HSD, $n = 4$). LPS increased ($P < 0.05$; $n = 4$) nitrite release at fetal P_{O_2} which was blocked by 1 μM dexamethasone (Dex) or 15 μM of the p44/42 mitogen-activated protein kinase (MAPK) inhibitor, PD98059. These effects were not observed at ambient P_{O_2}. p44/42 MAPK, an activator of NO synthesis, was inactive in untreated H441 cultures at ambient P_{O_2}, but was consistently active at fetal P_{O_2}; this was blocked on addition of 50 nM Dex. We conclude that fetal P_{O_2} values are requisite for NO production in fetal rat lung tissue and H441 epithelial cells. This activity is sustained by p44/42 MAPK phosphorylation and can be attenuated by dexamethasone.

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All procedures accord with current UK legislation.

Differential mechanisms of intracellular pH (pH_i) recovery following acid load to *in situ* bovine articular chondrocytes within different cartilage zones

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The regulation of pH_i by isolated articular chondrocytes is almost exclusively by the Na^+–H^+ antiporter (Wilkins & Hall, 1995). There are, however, significant variations in the environment of *in situ* chondrocytes with cartilage depth from synovial surface to bone. Here, we have investigated whether there are differences in the ability of chondrocytes within three cartilage zones (superficial, mid, deep; SZ, MZ, DZ) to recover their pH_i following an intracellular acid load.

Explants of bovine articular cartilage were obtained from metacarpophalangeal joints of animals freshly slaughtered at the local abattoir and cultured in Dulbecco's modified Eagle's medium (DMEM, 280 mosmol, pH 7.4). When required, explants were incubated with BCECF-AM (10 μM, 30 min, 37 °C) and chondrocytes within the zones imaged (Ex. 490/439 nm; Em. 535 nm) using a Photon Technology International ImageMaster system (Hall & Bush, 2001). pH_i was altered using the ammonium pre-pulse technique (Thomas, 1984) and recovery in fluorescence ratio studied in the presence of $NaHCO_3$ (23 mM) or its absence (NaCl substituted; both pH 7.4). There was no significant difference (ANOVA) to the change of fluorescence ratio following ammonium pre-pulse of cells within the three zones in the presence or absence of HCO_3 ($P = 0.68$; $n = 14$). In HCO_3-free media, recovery of pH_i (in units of change in fluorescence ratio $s^{-1} \times 10^5$) of cells within the MZ and DZ was rapid (32 ± 2 and 56 ± 2, respectively) and completely inhibited by ethylisopropyl amiloride (EIPA, 100 μM; -2 ± 6 and -1 ± 1, respectively). In contrast, cells within the SZ demonstrated no significant ($P > 0.05$; Student's unpaired t test) recovery of pH_i over the same time period in the absence of HCO_3, although in its presence recovery was markedly stimulated (49 ± 1; data are means ± S.E.M., $n = 3$).

These data suggest that chondrocytes within the SZ of bovine articular cartilage utilise a HCO_3-dependent system for pH_i recovery from acid load, whereas cells within the MZ and DZ of cartilage largely utilise the Na^+–H^+ antiporter. These differences in the mechanisms of chondrocyte pH_i regulation may relate to the environment experienced by the cells within the three cartilage zones. It is possible that this property of SZ chondrocytes has not previously been observed in isolated cell preparations, because they represent a relatively small (< 10 %) proportion of the total cell population.

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All procedures accord with current UK legislation.

Measuring the binding kinetics of myosin X PH domains at the plasma membrane by total internal reflection fluorescence microscopy

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The myosin superfamily contains over 18 family members (Hodge & Cope, 2000). Of these myosins, myosin X is unique in that it is the only myosin found in mammals that contains pleckstrin homology (PH) domains in its tail (Berg *et al.* 2000). PH domains are found in over 250 different proteins in the human genome and selectively bind to phosphoinositide phospholipids. Myosin X is expressed by several cell types, including cultured mouse myoblasts prior to their differentiation into myotubes (Wells *et al.* 1997). The PH domains of myosin X, of which there are three, are thought to bind to either $Pi(3,4,5)P_3$ or $Pi(3,4)P_2$, produced following phosphorylation of $Pi(4,5)P_2$ by Pi3kinase.

To measure the binding kinetics of the myosin X PH domains at the plasma membrane in cultured mouse myoblasts, a construct containing all three PH domains from myosin X fused to green fluorescent protein (PH123-GFP) was transiently expressed in cultured myoblasts, and single fluorophores were imaged in live cells using total internal reflection fluorescence microscopy (TIRFM). Single fluorescent spots (single PH123-GFP molecules) were identified using a set of D.I.S.H. criteria: they had Diffraction limited size, average Intensity of spots corresponding to single fluorophore intensity, spots disappeared (photobleached) in a Single step manner, and average Half-life was proportional to illumination intensity. Purified GFP molecules attached to a glass surface by antibodies were used as a control.

We found that single fluorophores disappeared suddenly either because they photobleached or because they detached from the membrane and diffused away rapidly. By varying illumination intensity we determined the rates of these different processes and we were able to measure the apparent binding and detachment rates of the pleckstrin homology domains. These experiments demonstrate that steady-state observation of single fluorophores in live cells gives information about binding and release kinetics of proteins at the plasma membrane.

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Effects of extracellular calcium and pH upon a chloride channel in the early distal convoluted tubule of the mouse

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We investigated the properties of basolateral Cl^- channels in the early distal convoluted tubule (DCT), using the cell-attached and excised configurations of the patch-clamp technique. Microdissected DCTs were isolated from collagenase-treated kidneys after mice were humanely killed. The pipette and bath solutions contained (mM): 140 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 Hepes (pH 7.4). We recorded one

channel of 9.4 ± 0.3 pS (n = 23, mean ± S.E.M.). In the conditions specified above, the reversal potential (E_r) of the I–V relationship is close to zero (2.0 ± 2.2 mV). Replacing 100 mM NaCl with sodium gluconate shifted E_r by 30.2 ± 3.7 mV (n = 4) towards the right, whereas substituting N-methyl-D-glucamine for Na^+ had no effect (−1 ± 1.9 mV, n = 5). This indicates Cl^- selectivity. We estimated the influence of extracellular Ca^{2+} and pH on channel activity by measuring the averaged current and NP_o on separate patches under various conditions of Ca^{2+} and pH. For this purpose, as the channel is inhibited at acid intracellular pH (Marvao & Teulon, 2000), the closed current level was assessed on the cell by inhibiting the channels via sodium acetate superfusion. The NP_o measured with zero Ca^{2+} in the pipette was 7.6 ± 1.6 (n = 13). It significantly increased to 33.2 ± 5.6 (n = 10) with 5 mM Ca^{2+} in the pipette. The influence of external pH was dramatic since NP_o significantly increased from 2.8 ± 0.1 (n = 9) at pH 6.4 to 44.7 ± 8.8 at pH 8.0 (n = 10). In contrast, the percentages of patches with active chloride channels were similar under the four conditions (71 and 62 % active patches at 0 and 5 mM Ca^{2+}; 58 and 54 % active patches at pH 6.4 and 8.0). Upon excision, it was possible to assess quantitatively the anionic selectivity of the channel in the early DCT despite frequent channel rundown. The channel had the relative permeability sequence $Cl^- > Br^- \sim NO_3^- \sim I^- > F^-$ (n = 4–5). The properties of the channel described here are compatible with the currently known properties of the ClC-K channels.

Student's unpaired t test was used. $P < 0.05$ was considered significant.

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All procedures accord with current National guidelines.

Carbachol-stimulated anion secretion in rat distal colon occurs predominantly through cystic fibrosis transmembrane conductance regulator (CFTR)

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The muscarinic receptor agonist carbachol is believed to evoke Cl^- secretion in rat colonic epithelial cells by elevation of intracellular Ca^{2+} (Greger *et al.* 1997) and although Ca^{2+}-activated Cl^- channels have been identified in colonic epithelial cell lines (Merlin *et al.* 1998) controversy exists over their presence/function in native colonic epithelia. Previous studies from this laboratory have shown that carbachol evokes Cl^- and HCO_3^- secretion in rat distal colon (Bellingham *et al.* 2002). Here we have examined the effects of both cAMP- and Ca^{2+}-activated Cl^- channel inhibitors on carbachol-evoked anion secretion in rat distal colon.

The terminal colon (5 cm) was removed from male Wistar rats killed by cervical dislocation. The mucosal portion was mounted in an Ussing chamber (area 0.5 cm^2) containing Krebs solution (mM: NaCl 119, KCl 4.7, $NaHCO_3$ 24.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.1) at 37 °C, gassed with 95 % O_2 and 5 % CO_2. Short-circuit current (I_{SC}) was recorded and measurements were taken from baseline to peak. All values represent means ± S.E.M. and Student's unpaired t test was used to test significance ($P < 0.05$).

Basal I_{SC} was 18.4 ± 5.2 μA cm^{-2} with a resistance of 102.3 ± 6.8 Ω cm^{-2} (n = 17). Enhancing cAMP levels by adding the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 100 μM basolaterally) increased I_{SC} by 45.0 ± 12.1 μA cm^{-2} (n = 3), suggesting tonic activation of cAMP-sensitive channels. Addition of the CFTR channel blocker diphenylamine-2-carboxylic acid (DPC, 100 μM apically) reduced basal I_{SC} by 9.9 ± 4.0 μA cm^{-2} (n = 3), whereas addition of the Ca^{2+}-activated Cl^- channel inhibitor 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS, 1 mM apically) had no effect on basal I_{SC} (n = 4). Furthermore, in the presence of DPC (100 μM apically) all three phases of the typical carbachol response (Fig. 1A) were significantly attenuated ($P < 0.05$, n = 3). However, in the presence of DIDS, carbachol-evoked I_{SC} was not significantly different from control (n = 4). Inhibition of endogenous cAMP production via the prostaglandin pathway, with the cyclo-oxygenase inhibitor indomethacin (10 μM basolaterally and apically), resulted in a markedly altered carbachol response (Fig. 1B, n = 11). Under these conditions, carbachol evoked a decrease in I_{SC} (−5.7 ± 2.5 μA cm^{-2}, n = 11).

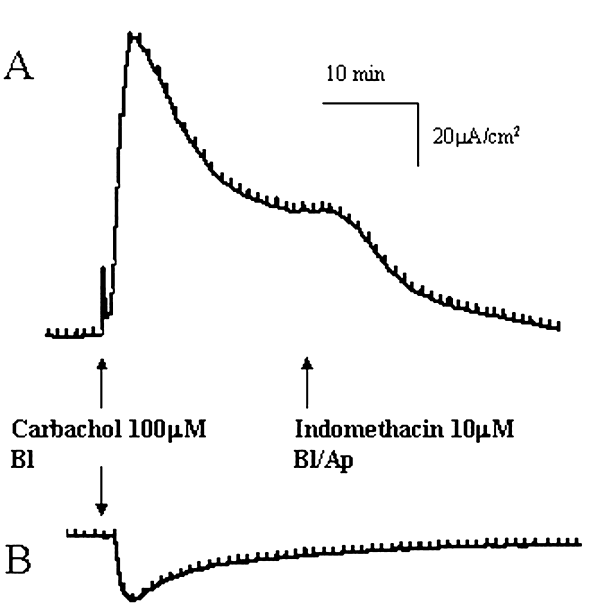

Figure 1. A, typical triphasic short-circuit current (I_{SC}) response to carbachol (100 μM, Bl) in rat distal colon (n = 17). B, monophasic I_{SC} response to carbachol (100 μM, Bl) in the presence of indomethacin (10 μM, Bl/Ap) in rat distal colon (n = 11). Bl = basolateral, Bl/Ap = basolateral and apical application.

The present results suggest that CFTR is the predominant Cl^- channel involved in carbachol-evoked anion secretion in the rat distal colon. These results support the findings of Strabel & Diener (1995) and provide further evidence against direct activation of Cl^- channels by carbachol in rat distal colon.

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All procedures accord with current UK legislation.

Effects of brefeldin-A upon the β-adrenoceptor-mediated control of apical Na^+ conductance (G_{Na}) in rat fetal distal lung epithelial (FDLE) cells

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If breathing is to be established at birth then the liquid secreted into the lung lumen during fetal life must first be removed from the potential air spaces. This occurs during the final stages of pregnancy and is driven by the β-adrenoceptor-mediated transport of Na^+ out of the alveolar space (Olver *et al.* 1986). It has been suggested that this stimulation of Na^+ transport involves the insertion of additional Na^+ channels into the apical plasma membrane by an exocytotic mechanism that is sensitive to brefeldin A (Ito *et al.* 1997). Recent work from this laboratory has shown that β-adrenoceptor agonists do increase G_{Na} in FDLE cells (Collett *et al.* 2002) and so we now explore the effects of brefeldin-A upon this response.

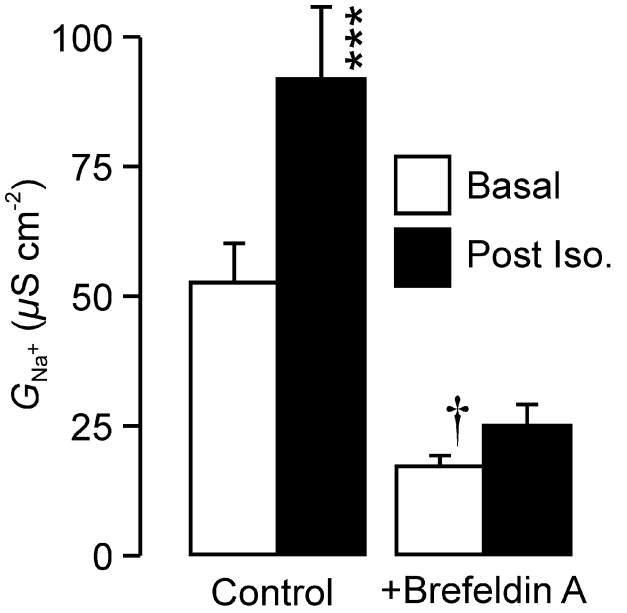

Figure 1. Time-mated pregnant rats were anaesthetised on the 20th day of gestation, their fetuses delivered by Caesarean section and immediately decapitated and the donor rats humanely killed without regaining consciousness. FDLE cells were then isolated from the fetal lung tissue as described previously (Collett *et al.* 2002). This acquisition of fetal lung tissue accords with current UK legislation. The cells were grown to confluence (~48 h) on Snapwell membranes (Costar) which were then mounted in Ussing chambers and I_{SC} measured. The cells were then basolaterally permeabilised (75 μM nystatin) so that G_{Na} could be estimated by measuring the amiloride-sensitive current evoked by imposing an inwardly directed Na^+ gradient upon the cells (Collett *et al.* 2002). Such experiments were undertaken using control and isoprenaline-stimulated cells that had either been maintained under control conditions or exposed to brefeldin-A (1 μM) for 30 min. Data are presented as means ± S.E.M. ($n = 6$). ***$P < 0.001$ *vs.* data derived from the unstimulated cells; †$P < 0.05$ *vs.* data derived from unstimulated control cells (Student's paired *t* test).

These data confirm (Collett *et al.* 2002) that isoprenaline-evoked increase in I_{SC} (basal, 7.9 ± 0.7 μA cm^{-2}; post-isoprenaline, 11.2 ± 0.9 μA cm^{-2}, $P < 0.02$) is normally associated with a rise in G_{Na} (Fig. 1). Exposing unstimulated cells to brefeldin-A (1 μM) caused a barely discernible rise in I_{SC} that developed over a period of ~5 min. The current then declined slowly, reaching a value that was 88.7 ± 3.6 % of its initial level ($P < 0.05$) after 30 min. Application of isoprenaline to brefeldin A-treated cells did not elicit a discernible change in I_{SC}. Data obtained after permeabilisation showed that this drug causes a fall in G_{Na} and blocks the isoprenaline-evoked increase in conductance (Fig. 1). Isoprenaline thus causes a brefeldin-A-sensitive rise in G_{Na} in FDLE cells (Ito *et al.* 1997; Collett *et al.* 2002).

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All procedures accord with current UK legislation.

Localisation of the two-pore domain K^+ channel TASK-2 in mouse kidney – RT-PCR of individual segments

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Potassium conductances and permeabilities are present in all nephron segments and serve various functions, such as K^+ excretion and the maintenance of cell potential and volume. TASK-2 potassium channel has two pore-forming domains in tandem in each monomer and is sensitive to extracellular pH. Messenger RNA encoding TASK-2 can be detected in RNA derived from whole kidney (Reyes *et al.* 1998). As a first step to understanding the role of TASK-2 in the kidney, we have attempted to localise TASK-2 mRNA by RT-PCR of individual nephron segments.

Adult male mice were anaesthetised with sodium pentobarbitone, 100 mg kg^{-1} I.P., and killed by decapitation. The kidneys were removed and divided into cortical and papillary sections by crude dissection, divided into 1 mm segments and digested with collagenase (0.5 mg ml^{-1}) at 37 °C in a shaking water bath for 30–60 min. Individual tubules were selected from the resulting tubule suspensions. Glomeruli and proximal tubules (PT) were identified by eye. Thick ascending limb (TAL) and cortical collecting tubule (CCT) segments were identified by the presence of mRNA for Tamm-Horsfall protein (THP) and aquaporin-2 (AQP-2), respectively. Amplification of endogenous β-actin mRNA was used as a positive control. Tubules were permeabilised with 0.5 % Triton X-100 and heating to 95 °C and reverse transcribed according to the manufacturer's instructions (Promega). Reverse-transcribed material was subjected to two rounds of PCR: the first with multiplex primers specific to TASK-2, AQP-2, THP and β-actin, and the second with nested primers. PCR products were analysed by gel electrophoresis, stained with ethidium bromide and visualised by UV illumination. Reverse transcriptions were also performed in the absence of enzyme to control for contaminating genomic DNA.

TASK-2 mRNA was identified in all nephron segments tested including those positive for THP and AQP-2 mRNA which correspond to TAL and CCTs, respectively. PCR products were not a result of contamination or amplification of genomic DNA.

In conclusion, TASK-2 is expressed along the length of the mouse nephron. This expression pattern contrasts with that of TASK-1 (Morton *et al.* 1999), which was localised to glomeruli and proximal tubules only. Given its location throughout the nephron it may be involved in the regulation of electrogenic solute uptake by the proximal tubule and/or K^+ excretion.

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Effect of W-7 on basolateral intermediate conductance potassium (IK_{Ca}) channels in human colonic crypts

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IK_{Ca} channels in epithelial and non-epithelial cells are exquisitely sensitive to Ca^{2+} despite the absence of Ca^{2+}-binding sites. Intracellular Ca^{2+} regulates IK_{Ca} activity by complexing with calmodulin, which is bound to the cytosolic carboxyl tail (Fanger *et al.* 1999; Khanna *et al.* 1999). Although calmodulin antagonists (e.g. W-7) might be expected to decrease IK_{Ca} activity, their effects are by no means clear, W-7 either having no effect (Fanger *et al.* 1999) or decreasing IK_{Ca} channel activity (Khanna *et al.* 1999) in human T-lymphocytes. The aim of the present study was to use W-7 to evaluate the role of calmodulin in the regulation of basolateral IK_{Ca} channels in human colonic crypt cells.

With ethics committee approval and informed consent, multiple biopsies were obtained from the distal colon of patients undergoing colonoscopy, who were free of mucosal disease. Intact crypts were isolated by Ca^{2+} chelation (Sandle *et al.* 1994) and used for patch clamp studies of the basolateral membrane in the cell-attached configuration (NaCl solution containing variable Ca^{2+} in bath and KCl containing 1.2 mM Ca^{2+} in pipette, holding voltage −40 mV). Data are shown as mean (± S.E.M.) single channel open probability (P_o). Differences between mean values were assessed by Student's unpaired *t* test.

In the absence of W-7, decreasing bath [Ca^{2+}] from 1.2 mM to 100 μM produced a reversible decrease in IK_{Ca} channel activity, whereas with 25 μM W-7 in the bath solution there was a high level of channel activity which was unaffected by changes in bath [Ca^{2+}] (Table 1). These results suggest that W-7 'clamps' IK_{Ca} activity at a high level set by the high initial bath [Ca^{2+}]. To explore this possibility, experiments were done with a low initial bath [Ca^{2+}], in which increasing bath [Ca^{2+}] from 100 μM to 1.2 mM produced a reversible increase in IK_{Ca} channel activity, whereas the presence of 25 μM W-7 ensured a high level of IK_{Ca} activity irrespective of bath [Ca^{2+}] (Table 2).

Table 1. Effect of W-7 on P_o with high initial bath Ca^{2+}

	1.2 mM	100 μM	1.2 mM
Control (n = 5)	0.612 ± 0.064	0.321 ± 0.087	0.500 ± 0.095
W-7 (n = 6)	0.645 ± 0.073	0.700 ± 0.042	0.646 ± 0.084
P	n.s.	< 0.003	n.s.

Table 2. Effect of W-7 on P_o with low initial bath Ca^{2+}

	100 μM	1.2 mM	100 μM
Control (n = 6)	0.271 ± 0.080	0.499 ± 0.090	0.128 ± 0.049
W-7 (n = 4)	0.661 ± 0.073	0.703 ± 0.046	0.711 ± 0.053
P	< 0.01	n.s.	< 0.0001

We conclude that W-7 enhances the activity of basolateral IK_{Ca} channels in human colonic crypt cells. This novel finding is inconsistent with the role of W-7 as a calmodulin antagonist, and raises the possibility that W-7 may increase intracellular Ca^{2+} concentration irrespective of extracellular Ca^{2+} concentration and/or enhance the sensitivity of human colonic IK_{Ca} channels to Ca^{2+}.

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

Electrophysiological evidence for KCNE3/KCNQ1 expression in human colonic crypts

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Secretory diarrhoea reflects the activation of electrogenic Cl^- secretion within the small intestine and/or colon. This involves activation of basolateral K^+ channels, which maintain a favourable electrochemical gradient for Cl^- exit across the apical (lumenal) membrane. At the mRNA level, KCNQ1 (KvLQT1) and KCNE3 (MiRP2) have been identified in mouse and human colon (Schroeder *et al.* 2000; Morton *et al.* 2001). When co-expressed in a heterologous system, these channels are stimulated by cAMP and inhibited by chromanol 293B. The present study sought functional evidence for KCNQ1/KCNE3 expression in human colonic crypts.

With ethics committee approval and written informed consent, biopsies were obtained during colonoscopy from the distal colon of patients who were free of mucosal disease. Intact crypts were isolated by Ca^{2+} chelation and stored at 4 °C for up to 24 h prior to use. Crypts were transferred to a perfusion chamber and immobilised by coating the base of the chamber with poly-L-lysine. Patch pipettes contained predominantly potassium gluconate and 0.24 mg ml^{-1} amphotericin. Whole-cell clamp was established within 10 min of seal formation. Data are given as means ± 1 S.E.M. and comparisons made with Student's unpaired *t* test.

Whole-cell currents were activated within 5 min by addition of 10 μM forskolin to the bath solution (current at +100 mV: 123 ± 21.4 pA control, 410 ± 79.2 pA after forskolin, $n = 3$). This forskolin-activated current was inhibited by 100 μM chromanol 293B and 1 mM $BaCl_2$. Prior to forskolin addition, currents were insensitive to chromanol 293B. In addition to these whole-cell recordings, small conductance channels of approximately 7 pS have been observed in cell-attached recordings with K^+-rich pipette solutions.

In conclusion, small conductance KCNE3/KCNQ1 channels are expressed in the basolateral membrane of human colonic crypts. These channels are stimulated by forskolin and inhibited by chromanol 293B and Ba^{2+} and may be expected to play a role in the pathogenesis of secretory diarrhoea.

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

P-Glycoprotein expression significantly increases the capability of human placental trophoblast cells to efflux xenobiotics

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We have previously shown that the trophoblast derived BeWo and JEG choriocarcinoma cell lines express MRP1 (multi-drug resistance related protein), whilst the JAr choriocarcinoma cell line and primary cytotrophoblast cells express both MRP1 and P-glycoprotein (P-gp, coded by the MDR1 gene) (Atkinson *et al.* 2000). The functional consequences of the absence of P-gp have also been determined and we have shown that the cyclosporin-sensitive accumulation of [^{3}H] vinblastine by cells expressing only MRP1 is higher than that by cells expressing both drug efflux proteins (Atkinson *et al.* 2001).

In the present study we tested the hypothesis that the increased cyclosporin-sensitive [^{3}H] vinblastine accumulation (i.e. reduced efflux) in BeWo cells is due to their lack of expression of MDR1 P-gp.

Using a retroviral vector based on the Moloney murine leukaemia virus we introduced MDR1 into BeWo cells by co-culture with the vector packaging cell line in transwell plates. MDR1 transduced cells (BeWo MDR) were then selected using 100 μg ml^{-1} of colchicine. To establish that transduction had been successful, Western blot analysis was performed using the F4 monoclonal antibody specific for human P-gp. The cells were then used in accumulation assays as previously described (Atkinson *et al.* 2001).

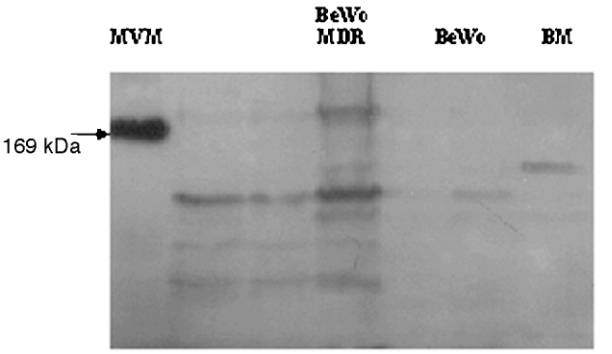

Figure 1. Expression of P-gp in native (BeWo, 13.6 μg) and transduced BeWo (BeWo MDR, 3.4 μg) cells. Microvillous (MVM, 10 μg) and basal (BM 15 μg) membranes of placental trophoblast are shown as +ve and −ve controls, respectively.

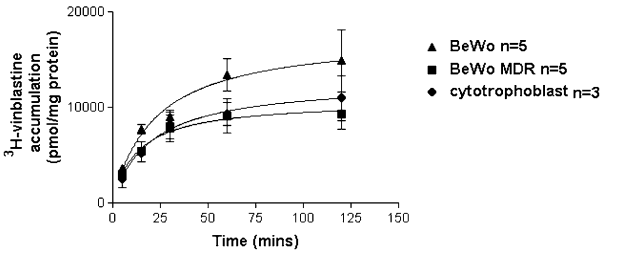

Figure 2. Cyclosporin-sensitive accumulation of [^{3}H] vinblastine in native BeWo cells and cells following transduction with MDR1. Accumulation by cytotrophoblast cells are included for comparison. Data are means ± S.E.M. ($P < 0.01$, two-way ANOVA)

BeWo MDR cells express P-gp, whilst, as expected, native BeWo cells show no expression of the protein (Fig. 1). Cyclosporin-sensitive accumulation of [^{3}H] vinblastine in BeWo MDR cells is significantly lower than in native BeWo cells and is very similar to that seen in cytotrophoblast cells which naturally express MDR1 (Fig. 2).

We conclude that the higher cyclosporin-sensitive accumulation in native BeWo cells resulting from a lower capacity to efflux substrate drugs is due to their lack of P-gp, and therefore that P-gp is important in xenobiotic handling by trophoblast cells. The expression of P-gp on the maternal facing plasma membrane of the placenta (Fig. 1, Atkinson *et al.* 2000) places it in an ideal position to protect the fetus from potentially harmful compounds in the maternal circulation.

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This work was funded by the MRC. MVM and BM were kindly donated by Dr P. Speake.

All procedures accord with current local guidelines.

Regulation of the AE2 anion exchanger by pH_i and pH_o requires highly conserved amino acids in the AE2 cytoplasmic N-terminal domain

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We recently reported that regulation of the murine Na^+-independent Cl^-–HCO_3^- exchanger, AE2, by pH requires amino acid residues 312–347 of its N-terminal cytoplasmic domain (Stewart *et al.* 2001). Initial pH-sensitive data employing hexa-alanine bloc mutations across this region prompted closer study of individual alanine substitutions in AE2 amino acid residues 336–347. AE2 function was measured as DIDS-sensitive $^{36}Cl^-$ efflux from *Xenopus* oocytes during variation of extracellular pH (pH_o) with clamped or unclamped intracellular pH (pH_i), or during variation of pH_i at constant pH_o. Statistical significance was assessed by Student's unpaired t test, where the level of significance was $P < 0.05$.

Wild-type (WT) AE2-mediated $^{36}Cl^-$ efflux was inhibited by acid pH_o, with a value of $pH_{o(50)} = 6.87 \pm 0.05$ (± S.E.M., $n = 36$), and was stimulated up to 10-fold by the intracellular alkalinization produced by bath removal of the pre-equilibrated weak acid, butyrate (40 mM; $n = 43$). Systematic hexa-alanine [$(A)_6$] bloc substitutions between aa 312–347 revealed that hexa-bloc mutation $A_{(6)}342$–347 exhibited the greatest acid shift in $pH_{o(50)}$ value of ~0.8 pH units ($n = 10$, $P < 0.05$). Two of the six $(A)_6$ mutants retained normal pH_i sensitivity of $^{36}Cl^-$ efflux, whereas the $(A)_6$ mutants 318–323 ($n = 10$), 336–341 ($n = 9$) and 342–347 ($n = 10$) were not stimulated by intracellular alkalinization ($P < 0.05$).

We further evaluated the highly conserved region between aa 336–347 by alanine scan and other mutagenesis of single residues. Significant changes in AE2 sensitivity to pH_o and to pH_i were found independently and in concert. Interestingly, activation of AE2 E346A by intracellular alkalinisation remained intact ($n = 25$, $P > 0.05$), but was abolished by the mutation E346D ($n = 9$, $P < 0.05$). In contrast, E346A acid shifted the $pH_{o(50)}$ value from WT AE2 by ~0.7 pH units ($n = 12$, $P < 0.05$).

Alanine substitution of conserved glutamate residues in the cytoplasmic N-terminal domain of two additional pH-sensitive polypeptides, cAE3 and the $AE1_{cyto}/AE2_{memb}$ chimera, confirmed the importance of these residues in the regulation by pH of other AE anion exchangers. We hypothesize that individual amino acid residues of AE2 aa 336–347 contribute to a local structure that interacts, directly or indirectly, with pH-sensor residues of the AE2 transmembrane domain. This regulatory interaction is probably conserved among members of the SLC4 bicarbonate transporter gene superfamily.

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All procedures accord with current National and local guidelines.

Oxygen tension may influence NHE1 protein expression in villous explants from human placenta

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We have previously demonstrated that Na^+–H^+ exchanger 1 (NHE1) protein is expressed and activity differentially regulated throughout gestation in the microvillous membrane (MVM) of the human placenta (Hughes *et al.* 2000). Furthermore, preliminary data have shown that NHE1 mRNA expression is lower in first trimester than term placenta (Lacey *et al.* 2001). Oxygen tension in the intervillous space of the placenta rapidly increases between 8 and 11 weeks of pregnancy, concomitant with the onset of blood flow through the spiral arteries (Jauniaux *et al.* 2001). Here we test the hypothesis that NHE1 protein expression in the placenta is regulated by oxygen tension. Villous explants were dissected from first trimester ($n = 2$, 11+6 and 10+5 weeks) and term ($n = 3$) placental tissue (5 mg wet weight) and maintained in explant culture on Netwell porous supports for 4–5 days, by which time term placental villous explants have undergone resyncytialisation (Siman *et al.* 2001). Explants were maintained in 3 or 21 % oxygen in 5 % carbon dioxide at 37 °C. On day 4–5 of culture, several explants were harvested, washed in PBS and protein homogenate prepared at 4 °C in PBS + 1.0 mM serine protease inhibitor. 20 μg protein homogenate from first trimester and term placental explants cultured at 3 and 21 % oxygen, together with MVM (positive control for NHE1; Hughes *et al.* 2000), were loaded on a 7 % reducing SDS-PAGE gel and electroblotted. Blotted membranes were probed with 1:100 mouse anti-human NHE1 primary monoclonal antibody (Becton Dickenson), followed by incubation with goat anti-mouse-HRP conjugated secondary antibody (1:1000) and detection by enhanced chemiluminescence. A band of 94 kDa representing NHE1 was observed in all samples. Intensity of NHE1 signal was calculated as optical density × area (Molecular Analyst Software, BioRad). In first trimester explants ($n = 3$), the median change in NHE1 protein expression, quantified as % change in optical density between 3 and 21 %, was −1.83 % (−63.08/+78.2 %, 95 % confidence interval), compared with term explants, $n = 5$, 27.85 % (−21.8/+106.1 %, 95 % confidence interval). We conclude that NHE1 protein expression in term human placenta may be affected by oxygen tension.

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The immunocytochemical localisation of anion exchangers (AE) in the reabsorptive duct of the human eccrine sweat gland

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Sweating carries with it a potential loss of salt and as NaCl is physiologically important, it must, therefore, be conserved. Functionally, the human eccrine sweat gland has two regions: a secretory coil, which produces an isosmotic primary sweat by the electrogenic movement of Cl^- and Na^+ ions, coupled osmotically to the movement of water, and a reabsorptive duct, which hypertonically reabsorbs NaCl from the primary sweat. The reabsorption of salt depends upon Na^+ being pumped from the duct cells into the interstitial fluid by a Na/K-ATPase on the basolateral membranes, while Na^+ moves passively into the cells via sodium channels sited only in the luminal membrane. ATP-dependent Cl^- (CFTR) channels allow Cl^- ions to follow down their electrochemical gradient. At low flow rates, sweat can be produced physiologically at concentrations as low as 5–15 mM NaCl. However, the electrochemical salt recovery mechanism cannot extract salt from the lumen when luminal Cl^- concentration falls below its electrochemical equilibrium across the apical membrane. Previous findings suggested that an additional mechanism for salt recovery at low flow rates may be driven by H^+ pumps associated with a Cl^-–HCO_3^- exchanger in the luminal membrane which could lower luminal Cl^- to the levels observed physiologically (Reddy & Quinton, 1994).

The presence of a H^+ pump has been demonstrated (Bovell *et al.* 2000), while that of a Cl^-–HCO_3^- exchanger has not been established. A family of anion exchangers (AE1, AE2 and AE3) have been cloned that functionally mediate Cl^-–HCO_3^- exchange and which are expressed in a variety of tissues. AE2 is expressed in many epithelia including kidney, while AE3 has been detected in the nervous system (bAE3) and cardiac muscle (cAE3), as well as in the gut. This study investigated the presence of an AE in the luminal cells of the human eccrine gland reabsorptive duct.

Frozen sections were obtained from skin biopsies donated, with informed consent and ethics committee approval, by male volunteers ($n = 6$). Immunocytochemical techniques employed antibodies, raised against AE2 and AE3. Appropriate negative controls were performed using pre-absorbed sera.

In this study, immunoperoxidase labelling demonstrated the presence of anion exchangers and showed a predominant localisation of AE2 at the apical plasma membrane of the ductal cells, suggesting that the AE2 is involved in exchanging Cl^- and HCO_3^- ions at slow sweat flow rates through the duct. However, the human sweat gland results differ from those in rat salivary duct cells (Park *et al.* 1999), where basolaterally located AE2 may, together with a Na^+–H^+ exchanger, control the intracellular pH and contribute to intracellular accumulation of Cl^-. Our results also demonstrate the localisation of AE3 in the cells of the reabsorptive duct, which is surprising. AE3 is normally found in either brain (AE3b) or cardiac tissue (AE3c), although it has been found in gut epithelia (Kudrycki *et al.* 1990). Further work is clearly required to clarify the role of these exchangers.

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

Blood glucose threshold for appearance of glucose in nasal secretions. Evidence for active glucose transport across human nasal epithelium?

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Glucose concentrations are low or undetectable in animal airway secretions (Barker *et al.* 1989). Sodium–glucose co-transporters in pulmonary epithelium, identified by RT-PCR in rat and mouse and by functional studies in sheep, could actively maintain this low glucose concentration (Barker *et al.* 1989; Lee *et al.* 1994). In healthy humans glucose is undetectable in airway secretions, but is present in secretions from those with hyperglycaemia (King *et al.* 2001; Meguer *et al.* 2001). The dynamic relationship between blood and airway glucose has not previously been studied.

Twelve non-diabetic volunteers with healthy nasal mucosa underwent repeated measurement of nasal glucose (NG) during elevation of blood glucose by the hyperglycaemic clamp technique. Nasal glucose was measured using glucose oxidase indicator sticks wetted by secretions during a 30 s application to the nasal mucosa under direct vision. Glucose was measured in arterialised whole blood using a glucose oxidase-based analyser (Analox, UK).

In six subjects blood glucose was raised to 12 mmol l^{-1} over 15 min. Glucose appeared in nasal secretions of all subjects within 20 min of commencing the infusion and 10 min of reaching the target blood sugar. NG decreased as the blood glucose fell on stopping the glucose infusion and was never higher than blood glucose. In a further six subjects blood glucose levels were raised from baseline by 1 mmol l^{-1} increments and held at each level for 15 min before NG measurement. The blood glucose level at which nasal glucose appeared was 8.2 ± 1.0 mmol l^{-1} (mean ± S.D.) (range 7.1–10.0).

Passive diffusion is likely to account for the rapid appearance of glucose in nasal secretions when blood glucose is raised. However, active transport must account for reabsorption of glucose against the concentration gradient as blood sugar falls. Our observation of a blood glucose threshold above which glucose appears in airway secretions is consistent with the presence of a saturable glucose transporter, such as a sodium–glucose co-transporter, actively reabsorbing glucose across human airway epithelium. Maintenance of glucose-free airway secretions may be important for pulmonary defence against infection.

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

The physiological roles of the KCl cotransporter in human cervical cancer cells

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The electroneutral KCl cotransporter (KCC) plays a role in cell volume regulation, transepithelial transport, and in the regulation of intracellular chloride concentration. Four KCC isoforms (KCC1–4) have been cloned. Human cervical carcinogenesis is accompanied by the upregulation of mRNA transcripts in KCC1, KCC3 and KCC4 (Shen *et al.* 2000). The up-regulation of volume-sensitive KCC activity is also associated with human cervical carcinogenesis. KCC works synergistically with ion channels for volume regulation of human cervical cancer cells. Truncation of the N-terminal 117 amino acids of KCC1 diminished KCC function and exhibited dominant negative phenotypes for KCC1, KCC3 and KCC4 (Casula *et al.* 2001). To study the physiological roles of KCC, we developed the stable transfection of N-terminal truncation of KCC1 mutant in human cervical cancer SiHa cell lines. KCC activities are studied using $^{86}Rb^+$ as a congener of K^+. In the wild-type cervical cancer SiHa cells, the KCC activity is nearly quiescent in normal physiological conditions but high transport rates are observed in response to hypotonic challenge, 1 μM staurosporine and 1 mM *N*-ethylmaleimide (NEM). In contrast, KCC1 mutant cervical cancer cells have KCl cotransport which is also quiescent in normal physiological conditions and is relatively refractory to hypotonic stimulation, and treatment with NEM or staurosporine. Basal intracellular Cl^- concentration in wild-type cervical cancer cells is 30 ± 2 mM (mean ± S.E.M., $n = 5$) estimated by $^{36}Cl^-$ equilibrium, and KCC1 mutant transfection significantly increases the intracellular Cl^- concentration to 46 ± 3 mM ($n = 5$, $P < 0.05$, Student's unpaired t test). Surprisingly, for wild-type cervical cancer cells, the swelling-activated Cl^- current is 70 ± 3 pA pF^{-1} ($n = 30$) at +100 mV and the current density is significantly decreased to 30 ± 2 pA pF^{-1} ($n = 20$; $P < 0.01$) for KCC1 mutant cervical cancer cells. In addition to altering the current amplitude, the activation rate of the swelling-activated Cl^- channel is also significantly decreased (wild-type: 0.8 ± 0.1 pA pF^{-1} s^{-1} at +100 mV, $n = 30$ *vs.* KCC1 mutant: 0.35 ± 0.08 pA pF^{-1} s^{-1}, $n = 20$, $P < 0.01$). Consistently, the swelling-activated taurine efflux rate constant decreases significantly from 0.06 ± 0.002 min^{-1} ($n = 6$) in wild-type cervical cancer cells to 0.032 ± 0.001 min^{-1} ($n = 6$) in KCC1 mutant cells ($P < 0.05$). KCC1 mutant cervical cancer cells display a slower growth curve. More importantly, the active forms of Rb and cdc2 kinase, two key regulators controlling cell cycle progression, are decreased in the KCC1 mutant cells. Considering the results overall, KCC plays an important role in the volume regulation and cell proliferation of human cervical cancer cells. Interestingly, reducing regulatory volume decrease (RVD) via inhibition of KCC1 also lowers the swelling-activated Cl^- currents, suggesting an interaction at either the signallings or membrane activity level for the process of RVD. In addition, overexpression of N-terminal mutant offers a unique non-pharmacological tool for functional inhibition of KCC gene products.

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Volume regulation by isolated bovine articular chondrocytes within gels

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An understanding of the processes involved in volume regulation of cartilage cells (chondrocytes) is of interest because changes can have a deleterious effect on matrix metabolism (Urban *et al.* 1993). There is growing interest in the use of 3-dimensional artificial gels (alginate, agarose) for suspending chondrocytes as this allows more precise control of the chondrocyte's environment, and prevents changes to phenotype which can occur (Guo *et al.* 1989). Here, we have compared the volume regulatory response of chondrocytes within gels to hyper- or hypo-osmotic challenge.

Articular chondrocytes isolated into DMEM (380 mosmol, pH 7.4) were obtained from cartilage explants taken from the bovine metacarpal-phalangeal joint of animals slaughtered at the local abattoir. Chondrocytes were entrapped in alginate (0.625 % w/v) or agarose (0.2 %) gels within 2 h and volume regulatory behaviour following osmotic challenges studied using fluorescence imaging of intracellular calcein (Hall & Bush, 2001). A hypotonic challenge (380–180 mosmol) caused rapid cell swelling and regulatory volume decrease (RVD) for cells in all gels (72 ± 15 % recovery within 10 min; for all results, data are means ± S.E.M., $n \geqslant 3$). In contrast, a hypertonic challenge (380–580 mosmol) caused rapid cell shrinkage but no regulatory volume increase (RVI) ($P > 0.05$, Student's unpaired t test). When cells were exposed to a hypotonic challenge and then returned to isotonic saline (the 'post-RVD/RVI protocol'), cells within agarose showed weak post-RVD/RVI (30 ± 5 % recovery in 10 min; $n \geqslant 3$). In contrast, the cells in alginate showed an overshoot in cell volume (140 ± 18 %). REV5901 (50 μM) and bumetanide (75 μM) blockers of the osmolyte channel and Na^+–K^+–Cl^- cotransporter, respectively, inhibited RVD and RVI of chondrocytes in alginate by 80 ± 10 and 95 ± 5 %.

The results show that post-RVD/RVI occurred mainly in chondrocytes suspended in alginate, but also in agarose gels. This response is not observed in freshly isolated chondrocytes (Kerrigan *et al.* 2002), suggesting that changes to chondrocyte physiology occur as a result of gel formation. The encapsulation of chondrocytes within gels is undoubtably a valuable experimental model, although these data suggest that this is not a benign process, and there may be longer-term consequences to chondrocytes which might not accurately model the behaviour of *in situ* cells.

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The role of the actin cytoskeleton in regulatory volume decrease (RVD) in bovine articular chondrocytes

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In response to a hypo-osmotic challenge, cartilage cells (chondrocytes) regulate their volume primarily by the activation of an 'osmolyte channel' (Kerrigan & Hall, 2000). The sensor(s) involved are unclear, and therefore in the present study we examined whether there is a role for the actin cytoskeleton in mediating chondrocyte RVD.

Full-depth cartilage explants were excised aseptically from bovine metacarpal phalangeal joints (obtained from the local abattoir) and chondrocytes isolated as described into DMEM (380 mosmol; Hall *et al.* 1996). For measurements of cell volume and $[Ca^{2+}]_i$, chondrocytes were incubated with fura-2 AM and imaged using a PTI Imagemaster system (Kerrigan & Hall, 2000). The actin cytoskeleton was labelled with Phalloidin-FITC and DNase-Texas Red for F- and G-actin, respectively, and visualised using a Zeiss CLSM510 confocal microscope. To examine the role of the intact actin cytoskeleton in RVD, chondrocytes were incubated with latrunculin B (2–5 μM for up to 30 min; Wakatsuki *et al.* 2001). Data were collected for 2 min (380 mosmol) under constant perfusion and then a hypo-osmotic challenge (220 mosmol) applied. There was no significant difference ($P > 0.05$; Student's unpaired t test) in either the fluorescence change (8.9 ± 1.5 %; 9.5 ± 0.8 %; data are means ± S.E.M.) or the time taken for maximal swelling between control and latrunculin B treated cells ($n = 5$ joints, 85 cells). In parallel, there was a transient rise in $[Ca^{2+}]_i$ that was not inhibited by latrunculin. RVD proceeded linearly with no significant difference ($P > 0.05$) in either the rate ($t_{½} = 5.2 \pm 1.6$ and 5.2 ± 0.7 min, respectively) or % cells responding (61 ± 6 and 61 ± 12 %) with complete volume recovery within 10 min. Confocal imaging of the actin cytoskeleton showed that under control conditions the F-actin was arranged cortically, whereas latrunculin addition disrupted this arrangement. The addition of REV 5901 (50 μM), a potent inhibitor of RVD, did not disrupt the actin cytoskeleton.

Rearrangement of the actin cytoskeleton might be a step in activating chondrocyte RVD. However, the present work suggests that when the cytoskeleton is disrupted, RVD is unaffected and thus an intact actin cytoskeleton is not necessarily required.

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Glucose-induced volume changes in α-cells isolated from rat pancreas

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Pancreatic α-cells secrete glucagon when plasma glucose concentrations are low. Glucagon secretion declines as glucose concentrations increase, but the mechanism by which secretion is inhibited is unknown. Recent work from our laboratory indicates that in pancreatic β-cells, glucose-induced changes in cell volume may help couple plasma glucose concentrations to insulin secretion (Miley *et al.* 1997). In the present study we have investigated the effects of glucose on the α-cell volume.

Rats were humanely killed by stunning and cervical dislocation. Islets were isolated from the pancreas by collagenase digestion, and dispersed into single cells using a Ca^{2+}-free medium. Cell volume was measured by video-imaging methods, and α-cells selected on the basis of volume, i.e. < 0.8 pl (Majid *et al.* 2001). Single α-cells were initially superfused with a control Hepes-buffered solution containing 5 mM glucose. The superfusate was then changed to a solution containing 20 mM glucose (isotonic replacement of mannitol) for 15 min. In this solution, relative cell volume increased to a maximum of 1.08 ± 0.03 (mean ± S.E.M.; $n = 8$). Cell volume recovered towards control values on return to the 5 mM glucose superfusate.

α-Cell volume was unaffected by superfusion with a solution containing 15 mM 3-*O*-methyl glucose (3-OMG; a non-metabolisable analogue of glucose) and 5 mM glucose. The volume changes (area under a volume *versus* time plot) in 20 mM glucose were compared by ANOVA with those with 20 mM 3-OMG or in time-matched controls (5 mM glucose). Cell volume increased in glucose compared with both 3-OMG ($n = 4$; $P < 0.05$) and the controls ($n = 6$; $P < 0.01$). There was no significant difference between the mean volume changes in 3-OMG and the controls ($P > 0.1$).

In conclusion, an increase in extracellular glucose concentration causes pancreatic α-cells to swell. These changes in cell volume may affect α-cell electrical activity, and help couple increases in glucose concentration to the inhibition of glucagon secretion.

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All procedures accord with current UK legislation.

Calcium dependency of RVD in ZR-75-1 cells

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RVD in ZR-75-1 cells, epithelial-derived human breast cancer cells, is HCO_3^- dependent and involves the parallel activation of the K^+ and volume-regulated anion channel (VRAC; Nicholl *et al.* 2001). Preliminary data indicated that the mechanisms of RVD were calcium (Ca^{2+}) dependent, although the exact nature is unknown. This study investigated the Ca^{2+} dependency of RVD in ZR-75-1 cells using the video imaging technique.

ZR-75-1 cells were bathed in isotonic solution at 37 °C for 10 min and allowed to equilibrate. They were subsequently exposed to a 23 % hypotonic solution for 15 min and then returned to isotonic solution for 12 min. Photographs were taken every minute and the area in pixels measured using Scion Image computer software. Cell volume was calculated and expressed as relative volume (mean ± S.E.M.) with respect to control values. The role of Ca^{2+} was investigated by exposure to Ca^{2+}-free solution and Ca^{2+} entry blockers. The significance of changes in cell volume during hypotonic exposure was tested using ANOVA followed by the Tukey HSD test.

In hypotonic solution control cells swelled to 1.27 ± 0.016 ($n = 8$) in 3 min and recovered to 1.03 ± 0.015 during the 15 min. The role of external Ca^{2+} was investigated using a Ca^{2+}-free hypotonic solution; cells swelled to 1.27 ± 0.004 in 4 min and after 15 min in hypotonic solution the volume was 1.26 ± 0.002 ($n = 3$). In the absence of extracellular Ca^{2+} the mean volume change was significantly less than the control ($P \leqslant 0.01$). The absence of RVD suggests a role for external Ca^{2+}.

The role of Ca^{2+} entry was investigated using 20 μM cadmium (Cd^{2+}), a non-specific voltage-gated Ca^{2+} channel blocker. Cells swelled to 1.27 ± 0.004 ($n = 5$) and after 15 min in hypotonic solution the volume was 1.19 ± 0.013. The type of Ca^{2+} entry pathway was investigated using 50 μM nifedipine, an L-type voltage-gated Ca^{2+} channel blocker. Cells swelled to 1.30 ± 0.018 ($n = 6$), and after 15 min in hypotonic solution the volume was 1.24 ± 0.048. Ca^{2+} entry was also investigated using 50 μM flunarizine, a specific T-type voltage-gated Ca^{2+} channel blocker. Cells swelled to 1.29 ± 0.029 ($n = 5$) and after 15 min in hypotonic solution the volume was 1.16 ± 0.038. In each case the volume change was significantly less than in the control ($P \leqslant 0.01$).

These results support preliminary experiments indicating that RVD in ZR-75-1 cells is Ca^{2+} dependent. RVD is inhibited in both the absence of extracellular Ca^{2+} and in the presence of Ca^{2+} entry blockers, Cd^{2+}, nifedipine and flunarizine. These results suggest that external Ca^{2+} enters into the cell via voltage-gated Ca^{2+} channels and favours entry via L-type voltage-gated Ca^{2+} channels.

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Modulation of airway morphogenesis in the pseudoglandular lung by endotoxin-induced interleukin 6 expression

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It is axiomatic that uterine infection raises the risk of preterm labour and postnatal respiratory disorder; however, recent work suggests that components of the inflammatory response may actively promote antenatal lung maturation (Moss *et al.* 2002). We sought to determine whether an inflammatory stimulus (*E. coli* lipopolysaccharide, LPS) impacts mesenchyme-epithelium differentiation through cytokine signalling pathways in the pseudoglandular lung.

Lung lobes (extracted following humane killing procedures) from gestation day 16 rat fetuses were mounted onto Whatman nucleopore track-etch filters (8 μm pore) floating on 2 ml of serum-free DMEM/Hams F12 and were maintained in variable O_2 incubators at either fetal (23 mmHg) or ambient (142 mmHg) P_{O_2}. Net change relative to 0 h culture in airway terminal bifurcation, oligonucleosome and lactate dehydrogenase release were used respectively as morphogenic, apoptotic and necrotic indices. TNFα, IL-1β and IL6 were measured by enzyme-linked immuno-absorbent sandwich assay.

Over 120 h, explants at fetal P_{O_2} showed a net 67.3 ± 2.7 % (mean ± S.E.M.) increase in airway terminal bifurcation, whereas those at ambient P_{O_2} showed a net 65.2 ± 6.20 % reduction. Addition of 0.5–50 μg ml^{-1} LPS did not alter bifurcation at ambient P_{O_2}, but caused a significant reduction at fetal P_{O_2} at 50 μg ml^{-1} LPS ($P < 0.05$, ANOVA; *post-hoc* Tukey's HSD, $n = 10$). No net shift in necrosis or apoptosis occurred, suggesting a regulated reduction in mesenchyme-epithelium differentiation. To manipulate LPS-evoked TNFα, IL6 and IL-1β release, explants were exposed to 50 μg ml^{-1} LPS plus 0.1–1000 nM of Zn^{2+}-conjugated thymulin at fetal or ambient P_{O_2}. At either P_{O_2}, thymulin significantly suppressed the release of TNFα at 0.1 nM ($P < 0.05$, ANOVA; *post-hoc* Tukey's HSD, $n = 5$), but did not alter IL6 or IL-1β release. This was associated with conserved mesenchyme mass without net change in necrosis or apoptosis.

We hypothesised that IL6 signalling via its nuclear factor, CCAAT enhancer binding protein (C/EBP)β, modulates fibroblast growth factor-9 (FGF-9) expression, a regulator of mesenchyme differentiation (Colvin *et al.* 2001). FGF-9 immunoprecipitate recovery was elevated over ambient P_{O_2} controls by 50 μg ml^{-1} LPS, LPS + 1 μM thymulin, 10 ng ml^{-1} recombinant rat IL6 and fetal P_{O_2}. C/EBPβ DNA binding activity matched the FGF-9 expression pattern and these effects were abrogated by rat IL6 antisense oligonucleotide. Explant immunostaining revealed an enhanced mass of FGF-9 and C/EBPβ positive mesenchyme in LPS+thymulin-treated explants over LPS alone. The demonstration that FGF-9 expression may be modulated by LPS-evoked IL6 signalling suggests that uterine infection during pregnancy is likely to alter the course of normal fetal lung morphogenesis.

Colvin, J. *et al.* (2001). *Development* **128**, 2095–2106.

Moss, T. *et al.* (2002). *Am. J. Respir. Crit. Care Med.* **65**, 805–811.

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All procedures accord with current UK legislation.

Amino acid transport by system A in the cat placenta

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The placental transport of amino acids by Na^+-dependent systems A and β is important for growth and development of the fetus (Sibley *et al.* 1999). There are no studies of amino acid transport by the cat placenta or other endotheliochorial placentas. We have recently developed a model to study taurine uptake by cat placental fragments and demonstrated characteristics of system β (Champion *et al.* 2001). Here we use the fragment model to measure methylaminoisobutyric acid (MeAIB) uptake to examine the functional activity of system A in the cat placenta.

Cat placentas were collected following natural litter production (one placenta per litter), with full cat welfare considerations in place. Dissection of the placenta was begun within 45 min of delivery. Fragments of the lamellar region (~10 mg wet weight), were isolated and tied to hooks in triplicate. MeAIB was used as a non-metabolisable specific substrate for system A. Uptake of [^{14}C]MeAIB was initiated by immersing the fragments in control Tyrode buffer (containing Na^+) or Na^+-free Tyrode buffer (choline replaced Na^+) containing 0.1 μCi ml^{-1} [^{14}C]MeAIB at 37 °C. Uptake was terminated by washing the fragments in an excess of control or Na^+-free ice-cold Tyrode buffer, after 5, 10, 15, 20 or 30 min to give a time course of uptake. Following lysis of the fragments in distilled water for 16–20 h, the lysate was counted for radioactivity as a measure of the labelled MeAIB transported into the placental fragments. The tissue was then transferred to 0.3 M NaOH to dissolve. A sample of each NaOH lysate was assayed to establish the protein content of each fragment. Na^+-dependent MeAIB uptake, attributable to system

A, was calculated as the difference between uptake in the presence and absence of Na^+.

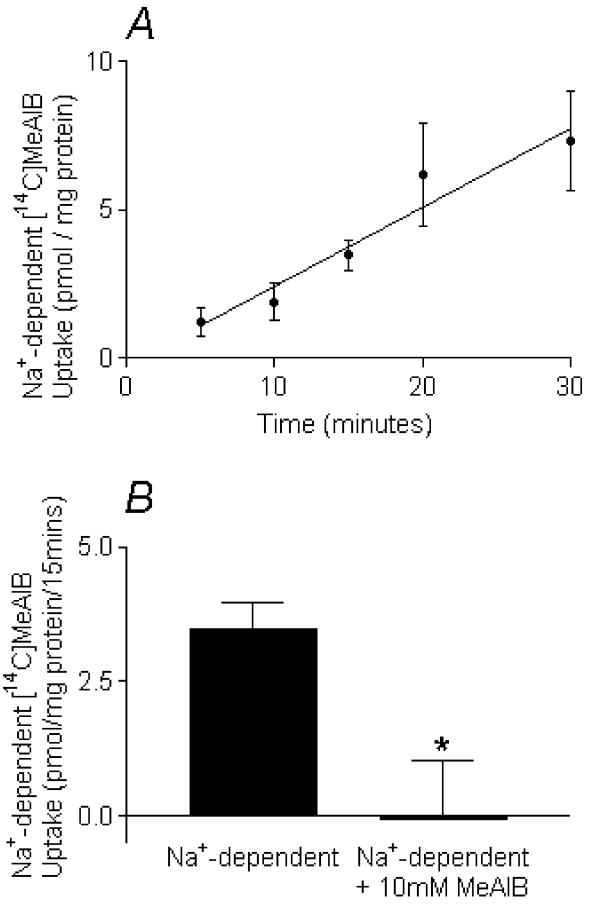

Figure 1. *A*, Na^+-dependent [^{14}C]MeAIB uptake into placental fragments was linear over 5–30 min, $r^2 = 0.48$, $P = 0.0001$; least squares linear regression. *B*, over 15 min, 10 mM cold MeAIB completely abolished the Na^+-dependent component of [^{14}C]MeAIB uptake. $^*P = 0.0136$ *vs.* control; paired *t* test. In the absence of Na^+, 10 mM cold MeAIB had no effect on [^{14}C]MeAIB uptake. Values are means ± S.E.M.; $n = 5$ placentas.

These results show that cat placental fragments exhibit system A-like transport characteristics. Thus system A as well as system β are likely to be important in provision of amino acids to the fetus by the cat placenta.

Champion, E.E. *et al.* (2001). *Placenta* **22**, 135P.

Sibley, C.P. *et al.* (1999). *Exp. Physiol.* **82**, 389–402.

All procedures accord with current UK legislation.

Relaxant effect of alkaloid, flavonoid and saponin fractions of *Pycnocycla spinosa* on rat isolated ileum

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Pycnocycla spinosa Decne. exBoiss. var. *spinosa* (Fam. Umbelliferae) is an essential oil-containing wild plant growing in central parts of Iran (Mozafarain, 1996). Hydroalcoholic extract of *Pycnocycla spinosa* has spasmolytic effects *in vitro* and anti-diarroheal actions *in vivo* (Sadraei *et al.* 2002). The aim of this research was to separate fractions of total hydroalcoholic extract of *P. spinosa* and screen for their spasmolytic activity to find out which fraction possesses the most antispasmodic activity. The hydroalcoholic extract of *P. spinosa* constituents includes alkaloids, flavonoids and saponins. These components were separated by a fractional liberation technique. Male Wistar rats were killed by a blow to the head followed by exsanguination. A portion of ileum was removed and suspended in Tyrode solution at 37 °C for contraction studies. An alkaloid fraction of *P. spinosa* in a concentration-dependent manner inhibited the ileum contraction induced by 80 mM KCl with an IC_{50} value of 4.5 ± 0.9 μg ml^{-1} (mean ± S.E.M., $n = 6$). With 10 μg ml^{-1} bath concentration the response to KCl was almost abolished. The inhibitory effect of the alkaloid fraction was reversible after washing the tissue with fresh Tyrode solution. The flavonoid extract also concentration dependently inhibited the tonic contraction to KCl ($IC_{50} = 12 \pm 2.1$ μg ml^{-1}, $n = 6$). The saponin fraction at relatively higher concentrations had an inhibitory effect on ileum contractions due to KCl. The inhibitory effect starts at 40 μg ml^{-1} and at 160 μg ml^{-1} about 18 % of the contraction still remained. The alkaloid extract of *P. spinosa* also reduced the tissue response to 5-hydroxytryptamine (5-HT, 1.28 μM) ($IC_{50} = 4.8 \pm 1.2$ μg ml^{-1}, $n = 6$) and acetylcholine (ACh, 320 nM) ($IC_{50} = 6.5 \pm 0.8$ μg ml^{-1}, $n = 6$) in a concentration-dependent manner. Equivalent volume of vehicle (ethanol) had no significant effect on KCl, ACh or 5-HT responses (ANOVA). From this study it can be concluded that alkaloid, flavonoid and saponin compounds in the total extract of *P. spinosa* contribute to the spasmolytic activity. As the alkaloid fraction has the most potent constituents and it can inhibit the ileum contractions induced by three different spasmogens, it is suggested that active materials in the alkaloid fraction should be further separated in order to identify the most active chemical structure, which could be used as an antispasmodic in gastrointestinal disorders such as diarrhoea.

Mozafarian, V. (1996). *A Dictionary of Iranian Plant Names*, pp. 443–444.

Sadraei, H. *et al.* (2002). *Phytotherapy Res.* (in the Press).

All procedures accord with current local guidelines.

A sodium-dependent mechanism responsible for thyroxine transport has been identified on the apical side of the lateral choroid plexus of the sheep

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The thyroid hormones (THs) are synthesized outside the brain and have important effects on the growth, development and metabolism of the central nervous system (Moore *et al.* 1973; Pickard *et al.* 1987).The entry of thyroxine (T_4) into the brain is via two main barriers, the blood–brain and the blood–cerebrospinal fluid (CSF) barriers, the choroid plexus (CP). However, the transport route of T_4 from the CSF compartments is still not fully understood. The aim of this research was to characterise the mechanism by which T_4 is transported from the CSF across the lateral CP. Therefore we investigated the effect of sodium replacement with choline in ACSF on ^{125}I-labelled T_4 transport at the apical side of the CP using the steady-state method with ^{125}I-T_4 in the ACSF to calculate CSF to blood

movement (R %) in the *in situ* perfused CP of the sheep. Sheep were anaesthetised with 20 mg kg^{-1} thiopentone (I.V.), heparinised and exsanguinated, and killed humanely. The brain was removed and the CP perfused with a modified Ringer solution via the internal carotid arteries. Both ventricles were then opened and the CP was superfused with ACSF containing 0.5 μCi ^{125}I-labelled T_4 and 10 μCi ^{14}C-labelled mannitol per 100 ml of ACSF. The steady-state uptake of ^{125}I-labelled T_4 was achieved after 1 h perfusion, then the ACSF was replaced with a low Na^+ ACSF. The results have shown that the control value for the ^{125}I-labelled T_4 after 1 h across CP was 0.012 ± 0.0013 (n = 9) and in the presence of low Na^+ was 0.006 ± 0.0005 (n = 3) (means ± S.E.M., $P < 0.05$, Student's unpaired t test).

These preliminary findings indicate that the significant reduction in the transport of T_4 from the CSF to the blood across the CP epithelial cells might be largely due to a Na^+-dependent mechanism present on the apical side of the CP cells, an important factor in T_4 transport into the CNS. Work is in progress to determine the mechanism of this effect.

Moore, T.J. *et al.* (1973). *Am. J. Physiol.* **225**, 925–929.
Pickard, R. *et al.* (1987). *Endocrinology* **121**, 2018–2026.

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All procedures accord with current UK legislation.

Rapid increase in urinary sodium excretion in response to aldosterone in the anaesthetised rat

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The classic mode of action of aldosterone involves nuclear transcription and synthesis of aldosterone-induced proteins over a period of hours to days. However, there is growing evidence to suggest that aldosterone, like other steroid hormones, may exert rapid effects via non-genomic mechanisms over a period of minutes. These rapid effects have been studied in a number of cell types, but there is little information about aldosterone's action on its major target site, the renal epithelium. *In vitro* studies have shown that aldosterone stimulates Na^+–H^+ exchange in MDCK cells within 2–4 min, via an intracellular Ca^{2+}-mediated mechanism (Gekle *et al.* 1997), but it is not known whether these effects are seen *in vivo*. Accordingly, the aim of this study was to determine whether aldosterone stimulates rapid changes in renal sodium excretion *in vivo*.

Under Intraval anaesthesia (100 mg kg^{-1} sodium thiopentone) male Sprague-Dawley rats received euvolaemic fluid replacement (2.5% dextrose, [^{3}H] inulin) of spontaneous urine output using a servo-controlled system (Burgess *et al.* 1993). After a 3 h equilibration period, control urine and plasma samples were taken for 1 h, following which half of the animals received aldosterone (42 pmol min^{-1}) for 1 h before returning to dextrose alone for the final hour. Animals were humanely killed at the end of the experiment.

Blood pressure (vehicle, n = 8, 90 ± 1 *vs.* aldosterone, n = 10, 103 ± 1 mmHg, means ± S.E.M.) and glomerular filtration rate (vehicle 0.70 ± 0.05 *vs.* aldosterone 0.81 ± 0.03 ml min^{-1} (100 g body wt)$^{-1}$) remained steady throughout the experiment. Urine flow rate (vehicle 15.1 ± 0.5 *vs.* aldosterone 15.4 ± 0.7 ml min^{-1} (100 g body wt)$^{-1}$ over hour of treatment, ANOVA, $P < 0.001$) and sodium excretion (Fig. 1) increased significantly during the period of aldosterone infusion.

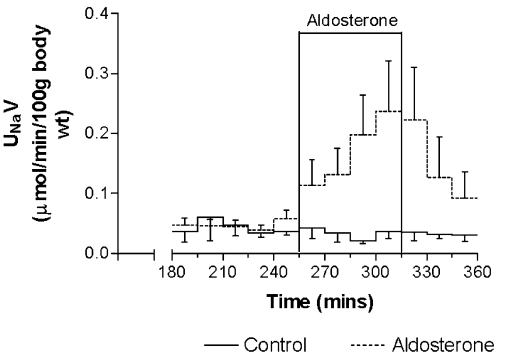

Figure 1. Urinary sodium excretion in vehicle (2.5% dextrose, n = 8) and aldosterone (42 pmol min^{-1}, n = 10) treated rats.

These changes were apparent within 15 min of the commencement of aldosterone infusion and had an equally rapid offset at the end of aldosterone infusion. The change in the sodium excretion rate from the control hour to aldosterone infusion hour was −12.9 ± 11.9% in vehicle infused rats compared with 404.7 ± 256.7 % in aldosterone infused rats (Mann-Whitney U test, $P < 0.01$). Potassium and osmolar excretion were not affected by aldosterone infusion.

These data show that aldosterone is able to induce a rapid increase in urinary sodium but not potassium excretion *in vivo*. This contrasts with its classic mode of action in which sodium excretion falls and potassium excretion increases over a period of hours to days. The mechanisms involved in this rapid response to aldosterone are unclear at this stage, but probably involve a non-genomic pathway.

Burgess, W.J. *et al.* (1993). *Clin. Sci.* **85**, 129–137.
Gekle, M. *et al.* (1997). *Am. J. Physiol.* **42**, C1673–1678.

All procedures accord with current UK legislation.

Simultaneous inhibition of the active and facilitated components of rat intestinal glucose absorption effectively abolishes all absorption in high stress perfusions

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We have previously reported that rat jejunal glucose absorption comprises an active component mediated by SGLT1 and a facilitated component mediated by GLUT2 (Kellett & Helliwell, 2000). At high glucose concentrations, similar to the effective concentrations likely to be present at the apical membrane after a meal, the facilitated component is as much as three to five times greater than the active. At 50 mM glucose the ratio of the passive to the active component was 2.1. These results were obtained by low stress jejunal perfusion *in vivo* with low flow rate (0.75 ml min^{-1}) and zero pressure head. Under these conditions, most of the facilitated component was dependent on the active component. Here we report the relationship of the two components in high stress perfusions with high flow rate and pressure head.

Jejunal loops of male Wistar rats (from humanely killed animals), anaesthetized I.P. with 1.0 ml Hypnorm plus 0.4 ml

Hypnovel, were perfused *in vivo* with 50 mM D-glucose in Krebs-Henseleit buffer in the absence (control) or presence of inhibitor(s). The perfusate was recirculated at a rate of 7.0 ml min^{-1} and segmented with gas (95 % O_2/5 % CO_2) at a rate of 2.0 ml min^{-1}: the pressure head of the perfusate reservoir was 18 cm, so that the jejunum became distended. Absorption was measured by the luminal disappearance of glucose; $n = 4$ for all perfusions, values given as means ± S.E.M. and statistical significance assessed by paired *t* test. The control rate was 42.0 ± 3.7 μmol min^{-1} (g dry wt)$^{-1}$. Phloridzin (0.5 mM) inhibited absorption by 51 ± 4 % ($P < 0.001$), a value close to that reported by Debnam & Levin (1975) under similar high stress conditions. Cytochalasin B (0.2 mM) inhibited absorption by 49 ± 8 % ($P < 0.001$), demonstrating that the large phloridzin-insensitive component was mediated by a facilitative transporter, confirmed by Western blotting to be GLUT2. The ratio of the passive to active components was therefore close to 1. Phloridzin and cytochalasin B together inhibited absorption by 92 ± 7 % ($P < 0.001$).

The results provide additional support for our proposal that rat intestinal glucose absorption comprises a facilitated as well as an active component. They further show that in high stress perfusions the facilitated component is independent of the active component, in contrast to the situation in low stress perfusions. The sum of the two components determined by independent inhibition with cytochalasin B and phloridzin accounts within experimental error for all absorption. This finding was confirmed by the fact that simultaneous inhibition of both components abolishes all but a few per cent of absorption. Non-carrier-mediated absorption is therefore minimal.

Debnam, E.S. & Levin, R.J. (1975). *J. Physiol.* **246**, 181–196.
Kellett, G.L. & Helliwell, P.A. (2000). *Biochem. J.* **350**, 155–162.

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All procedures accord with current UK legislation.