PC36

The Effect of LY294002 and Forskolin on Short Circuit Current in H441 Cells

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In renal A6 cells, basal Na^+ transport is regulated by PI-3-Kinase (Blazer-Yost *et al.* 1998, Paunescu *et al.* 2000, Record *et al.* 1998). This study aims to identify the role of PI-3-Kinase in the regulation of dexamethasone (dex) induced Na^+ transport in bronchiolar H441 cells, and to investigate whether forskolin regulates Na^+ transport via a pathway dependent upon PI-3-Kinase.

H441 cells were cultured in insulin free RPMI medium. On reaching confluence, cells were seeded onto Snapwell inserts (~0.5 x 10^{-6} cells per insert). After 24 hours medium was removed and basolateral medium was replaced with insulin free RPMI medium prepared using dialysed serum and containing dex (200nM) as the only hormone. The cells were grown at apical air interface for 8 days before being mounted in Ussing chambers.

The basal short circuit current (I_{sc} of monolayers was 33.08 ± 3.01μAcm^{-2} (n=7 all data shown as mean ± SEM) with transepithelial resistance (R_t of 214.51 ± 9.13 Ωcm^{-2} (n=7). This I_{sc} value is similar to that seen in H441 cells cultured in the presence of insulin 37.0 ± 2.8 μAcm^{-2} (n=7) (Ramminger *et al.* 2004) indicating that, in contrast to A6 cells, insulin is not required to regulate dex induced Na^+ transport. However, addition of the PI-3-Kinase inhibitor LY294002 (LY, 50μM) inhibited I_{sc} by 61.6 ± 4.4% after 55 mins). The addition of forskolin (10μM) stimulated I_{sc} with a peak (133.3 ± 15.9%) reached after ~20 mins, an effect which was still present when added 10 mins after LY. The amiloride sensitive I_{sc} (AS-I_{sc}) of monolayers receiving no drug treatment was 24.07 ± 3.07μA cm^{-2} (n=7). The addition of forskolin increased AS-I_{sc} to 35.37 ± 6.18μA cm^{-2} (ΔI_{sc} 11.30 ± 4.07μAcm^{-2} P<0.05 n=7) while addition of LY reduced AS-I_{sc} to 11.73 ± 2.81μAcm^{-2} (ΔI_{sc} -12.37 ± 2.77μAcm^{-2} P<0.05 n=7). The addition of forskolin after LY increased AS-I_{sc} to 33.10 ± 5.51 μAcm^{-2} (n=7 data analysed using Repeated measures ANOVA with Bonferroni Post-Hoc Test).

These data suggest that PI-3-Kinase maintains dexamethasone induced Na^+ transport in H441 cells, but, in contrast to that seen in renal A6 cells, insulin is not required to maintain the activity of this enzyme. Since inhibition of PI-3-Kinase does not block the response to forskolin it is likely that forskolin regulates Na+ transport via an independent pathway.

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

PC37

Modulation of the voltage-dependent chloride channel ClC-2 by short chain fatty acids.

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Colonic electroneutral NaCl absorption mechanism needs apical membrane Na^+/H^+ and $Cl^-/HCO3^-$ exchangers working in parallel. The basolateral exit pathway for Na^+ is via Na^+/K^+ ATPase. Recently, we have postulated that the basolateral voltage-gated chloride channel ClC-2, expressed in colonic surface epithelium, is the basolateral exit pathway for Cl^- (Catalán et al., 2004). Short chain fatty acids (SCFAs) are generated by colonic microflora and constitute the main source of energy for colonocytes. SCFAs are known to increases NaCl absorption in mammalian colon, but the molecular mechanism remains unknown. We have hypothesized that ClC-2 channel activity might be regulated by SCFAs. We have now tested whether intracellular SCFAs affect ClC-2 function. We have used the recombinant guinea-pig ClC-2 transiently expressed in HEK-293 cells and assayed by the whole-cell recording mode of the patch-clamp technique. In the presence of 35 mM intracellular Cl^- (100 mM gluconate) hyperpolarisation activated ClC-2 channel with a $V_{0.5}$ (voltage necessary to reach 50 % of maximal activation) of -108 ± 4 mV and a slope factor of -24 ± 1 mV (n=8). The reversal potential (Erev) for this current was identical to ECl (-36 mV) indicating perfect selectivity of Cl^- over gluconate. The same experiment was repeated replacing all gluconate with butyrate. Erev was not altered by butyrate replacement, indicating that butyrate does not permeate that channel. A blockade of the current by intracellular butyrate was apparent. This blockade was concentration- (IC_{50} 25 mM) and voltage-dependent, and was rather small at physiological (>-80 mV) potentials. The activation curve was shifted to more positive potential, with a $V_{0.5}$ of -80 ± 2 mV and a slope factor -18 ± 1 mV (n=5). This effect of butyrate is similar in magnitude to that obtained after replacing gluconate with Cl^-. The effect of butyrate on $V_{0.5}$ was concentration-dependent and could be described by a Hill equation with nH of 2 and KD 22 mM. SCFAs acetate and propionate also shifted the voltage-dependence of ClC-2, but other organic acids (glutamate and lactate) did not affect ClC-2 activity. We do not know if the activity of basolateral ClC-2 channels might be important for transport of SCFAs across the basolateral membrane. Its activation by these compounds suggests that their possible involvement is worth investigating.

Catalán, M., Niemeyer, M. I., Cid, L. P., & Sepúlveda, F. V. (2004). Gastroenterology 126, 1104-1114.

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

PC38

Role of Protein Tyrosine Kinase in Fetal Sheep Lung Liquid Homeostasis

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Fetal lung liquid (LL) secretion is essential for normal lung growth but at birth this liquid is absorbed rapidly by the β-adrenergic action of adrenaline which activates ENaC, an amiloride blockable sodium channel on the apical surface of the pulmonary epithelium. Sodium is transported into the interstitium with water and other ions thought to follow passively thus clearing the lung lumen of liquid (Olver et al., 2004). We have previously shown, by using the inhibitor KT5720, that protein kinase A does not appear to be involved in the activation of fetal lung ENaC. To explore the possibility that another kinase is part of the intracellular signalling mechanism between beta receptor stimulation and ENaC activation, we performed experiments investigating the involvement of protein tyrosine kinase (PTK).

An *in utero* fetal sheep model, prepared under general anaesthesia (induction with 5% thiopentone and maintenance with 2% halothane) as previously described (Olver et al., 1986) was used. We measured net fetal LL volume change with an impermeant marker (125I human serum albumin) at rest, in the presence of genistein and after stimulation with adrenaline with and without genistein. Experiments were performed at gestations between 135 and 146 days (term 147 days). Adrenaline was infused intravenously at a set rate of 1μg/min and genistein was administered by dissolving in a small amount of DMSO and instilling into the LL to give a final concentration of about 2.6 mM.

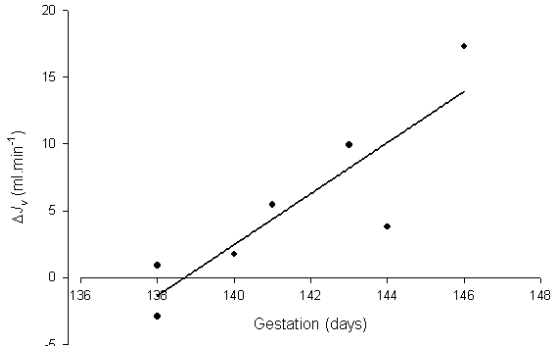
Genistein alone caused a gestation dependant inhibition of LL secretion (Figure), presumably by activating ENaC, since the effect is blocked by amiloride. Genistein did not block or inhibit the effect of adrenaline but rather enhanced its effect in causing LL absorption (Table).

These results are opposite to those of Niisato et al. (1999) who showed that cAMP stimulated sodium transport is inhibited by genistein in rat fetal distal lung epithelial cells grown in culture.

We conclude that PTK may produce an inhibitory effect on ENaC i.e. tending to keep it in the inactive state, although an effect on the fetal LL chloride secretory mechanism has not been excluded yet.

Effect of genistein on LL secretion prior to adrenaline stimulation. *P<0.05 compared to second control slope and the first adrenaline slope using Student paired t-test.

n=4	Control	Adrenaline	Control	Genistein	Adrenaline	Amiloride
Mean Jv (ml.hr-1)	13.25	2.16	9.89	-2.09	-10.72*	6.55
S.E.M.	4.37	4.21	3.02	5.67	2.91	3.16



Gestational effect on genistein on resting LL secretion. r = 0.87, P < 0.02 (ANOVA)

Niisato, N. et.al. (1999) *Am J Physiol* **227**, L727-L736.

Olver, R.E. et.al. (2004) *Ann Rev Physiol* **66**, 77-101.

Olver, R.E. et.al. (1986) *J Physiol* **376**, 321-340.

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

PC39

The effect of LPS on amiloride-sensitive Na^+ currents across H441 lung epithelial cells.

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Bacterial lipopolysaccharides (LPS) are potent inducers of pro-inflammatory cytokines via activation of nuclear factor– kappa B (NF–κB) and mitogen activated protein kinase (MAPK) signalling pathways [1]. Bacterial infections also modify the ion transport processes that control lung fluid homeostasis and this may contribute to the formation of pulmonary oedema [2]. Therefore, we investigated the effect of LPS on expression of the pore-forming α–subunit of the amiloride-sensitive Na^+ channel (ENaC) and amiloride-sensitive Na^+ transport across H441 human lung epithelial cells. We have used inhibitors of NF–κB and MAPK activation to examine the role of these signalling pathways in LPS mediated effects.

H441 cells were cultured at air interface on permeable supports for 7 days, mounted in Ussing chambers and bathed in physiological saline. H441 monolayers exhibited a mean resistance (R_t) of 240± 50 $\Omega.cm^{-2}$ Spontaneous short circuit current (I_{sc}) was measured by clamping transepithelial voltage (V_t) at zero. Amiloride-sensitive I_{sc} was calculated after application of 10μM amiloride to the apical chamber (IC_{50} = 0.9μM). As spontaneous

I_{sc} varied between batches of cells, treatments were carried out on paired monolayers from the same batch of cells and results analysed using Student's paired t–tests. Data are presented as mean±SEM.
Incubation of cells for 16 hours with 15μg.ml^{-1} LPS (from Ps. aeruginosa) caused a significant reduction in amiloride-sensitive I_{sc} from 14 ± 2 to 7 ± 2 μA.cm^{-2}, p=0.01, n=6). There was no change in amiloride-insensitive I_{sc} or resistance of the monolayers. The LPS-induced reduction in I_{sc} was associated with a decrease in αENaC mRNA and protein abundance. Treatment of cells with 5mM sulphasalazine (SAS), an inhibitor of NF–κB activation, or 25 μM caffeic acid (CAPE), an inhibitor of NF–κB nuclear translocation, prior to addition of LPS did not restore amiloride-sensitive I_{sc} to control levels. However, pre-treatment with 20μM PD98059 (to inhibit MAPK activity) resulted in an increase in amiloride-sensitive I_{sc} that was significantly higher than cells treated with LPS alone (26 ± 3.0 μA.cm^{-2}, 13 ± 5.0 μA.cm^{-2} respectively, p⟨0.05 n=4). Furthermore, αENaC protein levels were more abundant in these cells compared to those treated with LPS alone.

Our data indicate that LPS decreases amiloride-sensitive Na^+ transport across H441 human lung epithelial cells by reducing αENaC abundance and that this effect is mediated by a pathway that involves activation of MAPK.

Guha M and Mackman N (2001) *Cellular Signalling* **13**, 85-94.

Rezaiguia S *et. al.* (1997) *Journal of Clinical Investigation* **99**, 325-335.

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

PC40

Function and distribution of human EGFP-labelled αENaC subunits in H441 lung epithelial cells.

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The amiloride–sensitive Na^+ channel, found in the apical membrane of polarized epithelia form the rate–limiting step for Na^+ reabsorption and fluid homeostasis [1]. We have produced a stably transfected clone of the H441 cell line that contain low levels of enhanced green fluorescent protein (EGFP)–labelled human αENaC subunit. We have confirmed that the appropriate EGFP–αENaC RNA and protein are expressed in the generated cell line. We have also determined the subcellular localization using confocal microscopy and measured functional amiloride-sensitive Na^+ transport in the presence and absence of forskolin. H441 cell monolayers were cultured on permeable supports for 7 days at air interface, mounted in Ussing chambers and spontaneous short circuit current (I_{sc}) was measured by voltage clamping the monolayers at zero. Statistical analysis was carried out using Student's t-test where p values of < 0.05 were considered significant. Data are presented as mean ± SEM.
Cells expressing EGFP–αENaC (αC3) exhibited an I_{sc} of 16.3 ± 0.7 μA cm^{-2} compared to 6.4 ± 0.7 μA cm^{-2} in cells expressing EGFP alone (p<0.001, n=3). Forskolin stimulated an increase in I_{sc} in clone αC3 of 7.0 ± 2.1 μA cm^{-2} which was significantly greater than cells expressing EGFP alone (0.09 ±0.08 μA.cm^{-2}, p<0.05, n=3). Application of 10μM amiloride to the monolayers demonstrated that amiloride–sensitive I_{sc} was also significantly higher in αC3 cells than in those transfected with EGFP alone (10.7 ± 0.84 and 3.0 ± 0.4 μA.cm^{-2} respectively p=0.001, n=3). Composite images from 10 μm sections of forskolin treated cells showed increased EGFP fluorescence in the upper (apical) compartment of the αC3 clone which was not evident in cells expressing EGFP alone.
Taken together, these data show that stable expression of human EGFP–αENaC subunits in H441 cells results in functional amiloride–sensitive Na^+ channels. Treatment with forskolin increased function and was associated with increased fluorescence in the upper compartment of the cell.

Schild L and Kellenberger S (2001). *Adv Exp Med Biol* **502**, 305-14.

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

PC41

PKA regulation of mouse TASK-2

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TASK-2 is a two-pore domain K^+ channel (K_{2P}) sensitive to extracellular pH; currents are maximal at alkaline pH but inhibited as pH decreases (Reyes *et al.*, 1998). TASK-2 is found principally in epithelial tissues and in the kidney is located in the basolateral membrane of proximal tubule cells where it is coupled to HCO_3^- transport (Warth *et al.*, 2004). Mouse TASK-2 possesses one putative PKA concensus site (S266, PROSITE). Our aim was to investigate whether (de)phosphorylation of this residue regulates channel activity.
Wild-type (WT) mTASK-2 cDNA was subcloned into the bicistronic vector pIRES-CD8 (Invitrogen). Mutations were generated by PCR and confirmed by sequencing (Lark). Chinese hamster ovary (CHO) cells were transfected using Fugene (Roche). After 24 to 72 hours CHO cells expressing the CD8 antigen were identified with anti-CD8 magnetic beads (Dynal). Whole-cell currents were recorded in mammalian Ringer containing (in mM); NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 5, PIPES 5, pH 7.8. Pipette solution contained (in mM); K-gluconate 135, KCl 10, $MgCl_2$ 5, $CaCl_2$ 611 μM, EGTA 5, HEPES 10 and K_2-ATP 5. Free Ca^{2+} activity was 20 nM (React program, Godfrey Smith, Glasgow University). Fluoride was added by replacing 20 mM K-gluconate with KF. Channel rundown was measured as the channel activity remaining after 2 mins. Results are given as means ± SEM, compared using the unpaired Students *t*-test.
mTASK-2 currents were outwardly rectifying and pH sensitive. With time channel activity decreased such that after 2 mins of whole-cell recording only 56.7±0.03% (n=7) of channel activity remained. In the presence of 20 mM F, a non-specific inhibitor of phosphatase activity, channel rundown was abolished;

98.8±0.03% channel activity remained after 2 mins. The PKA inhibitor, H89 (5 μM, in the presence of F) caused a reversible decrease in channel activity; 75.9±0.03% WT channel activity remained after 2 mins (n=8). S266A and S266D mutants expressed pH sensitive currents not different from WT. After 2 mins, 72.2±0.02% (n=11) and 74.7±0.12% (n=4) channel activity remained, respectively.
The C-terminus of mTASK-2 is 252 amino acids long and encodes one concensus PKA site at residue S266. The inhibition of rundown by F and rundown caused by H89 suggest that PKA has a role in the regulation of TASK-2; normal channel activity is maintained by PKA-mediated phosphorylation. Given that both S266 mutants express as WT, PKA must be acting either at a site other than S266 or on an, as yet unidentified, accessory subunit.

Reyes, R. et al (1998), J. Biol. Chem. 273, 30863-30869.

Warth, R. et al (2004), PNAS 101, 8215-8220

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

PC42

Subcellular distribution of the ClC-2 chloride channel in cell lines

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ClC-2, a plasma membrane chloride channel, is widely distributed in different organs and tissues. Functionally, it has been proposed to play roles in transepithelial fluid transport and chloride homeostasis in neurones. In the intestinal epithelia and polarised cell lines, the channel is located in the basolateral membrane, presenting in addition an intracellular vesicular pattern of expression. The goals of the present study were, 1) to identify the intracellular pool in which ClC-2 is located using markers of the secretory and endocityc pathways, and 2) to evaluate the dynamic of the protein in the plasma membrane.
HEK-293 cells were transiently transfected with hClC-2/EGFP (Green Fluorescent Protein, EGFP, in the C-terminus) or hClC-2/HA (Hemagglutinin peptide epitope, HA, in an extracellular loop between the L and M transmembrane domains). Both constructs behaved like the wild type channel: HEK-293 cells transiently transfected with either construct displayed typical slow currents activated by membrane hyperpolarization; and destination experiments in MDCK cells revealed the usual basolateral localisation.
Confocal microscopy studies with markers of early and recycling endosomes (syntaxin-13 and transferrin receptor), late endosomes and lisosomes (lysotracker), endoplasmic reticulum (pEYFP-ER) and Golgi apparatus (pEYFP-Golgi), did not show any significant colocalization with the channel protein. All these experiments were repeated using at least three different cell cultures.
To determine the dynamics of the channel protein at the plasma membrane surface, we treated the HEK-293 transfected cells with beta-cyclodextrin (βCD) and followed the localisation of the constructs by confocal microscopy or surface protein biotinylation. Using either construction, incubation with 10 mM βCD during 30 min at 37°C increased significantly the expression of channel at the cell surface. The observed increase in biotinylation experiments was 2.6±0.2-fold (mean±SD, $p<0.05$, 3 experiments, 2-3 replicates in each experiment). Surprisingly, in parallel patch-clamp experiments treatment with βCD decreased the current mediated by ClC-2 by 53±10% (n=5, $p<0.05$).
Based on present results, we hypothesize that ClC-2 could be present in an intracellular "non classical" vesicular compartment, and that cholesterol depletion-sensitive dynamic traffic towards, and retrieval from, the cell surface accounts for the membrane ClC-2 expression in the basal state.

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

PC43

Coupling cytoskeletal rearrangement to GLUT2-trafficking in rat small intestine

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We have previously shown that when rat jejunum is challenged with high concentrations of glucose, the facilitative transporter GLUT2 is mobilised during Na^+/coupled transport and inserted into the brush border membrane (Kellett and Helliwell, 2000). GLUT2 then affords the major route of absorption so that absorptive capacity is precisely regulated to match dietary intake. In Caco-2 cells, Na^+/glucose cotransport induces cytoskeletal rearrangement caused by myosin II regulatory light chain (myosin II RLC_{20}) phosphorylation in the perijunctional actomyosin ring (Turner *et al.*, 1997). We report here that GLUT2 insertion into the brush border membrane of rat jejunum requires myosin II RLC_{20} phosphorylation.
All animal procedures conformed to the UK Animals (Scientific Procedures) Act, 1986. Male Wistar rats were anaesthetised by an I.P. injection of a mixture of 1.0 ml Hypnorm (Janssen Animal Health) and 0.5 ml Hypnovel (Roche Diagnostics) per kilogram body weight. Fed rats were perfused with 75 mM glucose *in vivo* to insert GLUT2 into the brush border membrane. Myosin light chain kinase (MLCK) phosphorylates myosin II RLC_{20} inducing actomyosin contraction. ML-7 (5 μM), a cell permeable and potent inhibitor of MLCK inhibited total glucose absorption by ∽ 50 % from a control rate of 38.0 ± 0.5 to 20.2 ± 0.8 μmol min^{-1} (g dry weight)$^{-1}$ ($p < 0.001$, $n = 9$). Selective inhibition of the GLUT2 component with phloretin (1 mM) demonstrated that ML-7 inhibited the GLUT2 component from a rate of 20.2 ± 0.8 μmol min^{-1} (g dry weight)$^{-1}$ in the presence of ML-7 (5 μM) to 10.5 ± 0.6 μmol min^{-1} (g dry weight)$^{-1}$ in the presence of ML-7 and phloretin ($p < 0.001$; $n = 9$). This effect was correlated to a 60.3 ± 1.8 % diminution in apical GLUT2 levels ($p < 0.001$; $n = 3$); SGLT1 levels were unaffected. Western blotting of total and phosphorylated myosin II RLC_{20} showed a 3-fold reduction in this ratio in response to ML-7 and an ∽ 4.5-fold reduction in the absence of glucose.
We conclude that initiation of Na^+/glucose cotransport stimulates Ca^{++} entry through $Ca_V1.3$ in the brush border membrane (Morgan *et al.*, 2003) resulting in myosin II RLC_{20} phosphorylation and subsequent actomyosin contraction. The ensuing cytoskeletal rearrangement seems essential to increase the absorptive capacity of the small intestine for glucose via insertion of GLUT2 into the brush border membrane.

Kellett G.L. and Helliwell P.A. (2000) *Biochem. J.* **350**, 155-162.

Turner J.R. *et al.* (1997) *Am. J. Physiol. Cell Physiol.* **273**, C1378-C1385.

Morgan E.L. *et al.* (2003) *Biochem. Biophys. Res. Commun.* **312**, 487-493.

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

PC44

Activation of the human α-ENaC gene promoter by cortisol

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At birth, the lung develops a Na^+ absorbing phenotype that is vital for lung function and which depends upon epithelial Na^+ channels that are formed from three subunits (α-, β- and γ-ENaC). The development of this phenotype requires prior exposure to circulating glucocorticoids but the physiological basis of this dependence is not clear (Olver *et al.*, 2004). However, dexamethasone can activate the α-ENaC promoter with no apparent effect upon β- and γ-ENaC and, since α-ENaC is the only subunit vital for lung function, it has been suggested that glucocorticoid-evoked α-ENaC expression may be important to the development of the pulmonary Na^+ absorption (Sayegh *et al.*, 1999). However, the concentrations of cortisol, the natural glucocorticoid, needed to elicit this response are unknown which makes it difficult to assess the significance of glucocorticoid-evoked α-ENaC transcription to lung maturation *in utero*. In the present study we therefore explored the extent to which cortisol could activate a luciferase-linked reporter construct (KR-1-*p*GL3) containing a 2.2 kb upstream region of the human α-ENaC gene (Sayegh *et al.*, 1999; Richard *et al.*, 2004). Whilst our data (Fig 1) show clearly that cortisol activates this construct as effectively as dexamethasone, the EC_{50} value was 108 ± 13 nM (estimated by least squares regression), which is ~20-fold greater that our recently published value for dexamethasone (~5 nM, see Richard *et al.*, 2004). Assays (Gitau *et al.*, 2001) of blood collected from the umbilical vein have shown that cortisol levels reach ~0.7 μM after normal vaginal delivery and ~0.3 μM after elective Caesarean section. The cortisol levels experienced during birth are therefore sufficient to cause maximal activation of the α-ENaC promoter (Fig 1).

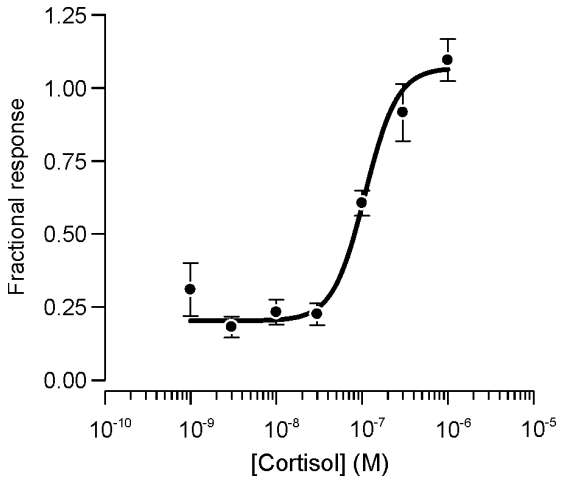

Fig. 1. H441 cells transfected with the KR-1-pGL3 construct were incubated (18 h) in dialysed (i.e. hormone-free) bovine serum supplemented with cortisol (3 nM – 1 μM), KCl, (5 mM) and glucose (5 mM). Cortisol-evoked (16 h) increases in transcriptional activity (see Richard et al., 2004) were determined and plotted (mean ± s.e.m., n = 3) against the concentration of cortisol used. All data are normalised to the increase in transcriptional activity evoked by 0.2 μM dexamethasone.

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

PC45

Forskolin desensitises the human α-ENaC gene promoter to dexamethasone

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Glucocorticoids activate the promoter region of the epithelial Na^+ channel α subunit (α-ENaC) gene (e.g. Sayegh *et al.*, 1999; Richard *et al.*, 2004) and, in the lung, this response may be important to the development of the Na^+ absorbing phenotype that normally becomes apparent at the time of birth. This transcriptional response is mediated by glucocorticoid response elements in the upstream region of the α-ENaC gene and, although this gene region also contains putative cAMP response elements, sig-

nalling via the cAMP / PKA-dependent pathway does not seem to evoke transcription (e.g. Sayegh *et al.*, 1999). However, the possibility that signalling via cAMP / PKA may modify the transcriptional response to glucocorticoids has not been explored and so the present study explores the effects of forskolin, an alkaloid that evokes cAMP formation, upon the glucocorticoid-induced activation of a reporter construct (KR-1-*p*GL3) containing the 2.2 kb upstream region of the human α-ENaC gene (Sayegh *et al.*, 1999; Richard *et al.*, 2004). Initial studies confirmed that forskolin does not induce transcription or modify the response to a maximally effective concentration of dexamethasone (Fig 1A). However, experiments that explored the effects of a range of dexamethasone concentrations (Fig 1B) showed that forskolin shifted the concentration - effect curve for dexamethasone to the right (control EC_{50}: 3.6 ± 0.5 nM; forskolin EC_{50}: 13.0 ± 2.5 nM, $P < 0.001$, Student's t test). This suggests strongly that signalling via cAMP / PKA may desensitise the α-ENaC promoter to dexamethasone.

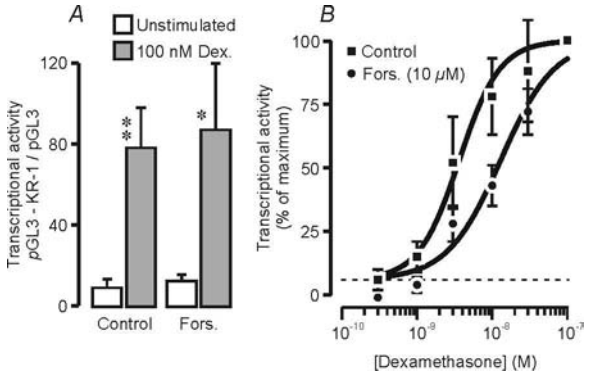

Fig. 1. H441 cells were transfected with the KR-1-pGL3 construct or the empty, pGL3 vector and h) before transcriptional activity was assayed (Richard et al., 2004). (A) A strictly paired experimental design (n = 5) was used to explore the effects of forskolin (10 μM) upon transcriptional activity (mean ± s.e.m) in unstimulated cells and cells exposed to a maximally effective concentration of dexamethasone (100 nM); asterixis denote significant responses to dexamethasone (Student's paired t test, * P <0.05; ** P < 0.02). (B) Concentration-response curves showing the dexamethasone induced activation of KR-1-pGL3 in control and forskolin-treated (10 μM) cells (n = 5). The sigmoid curves were fitted to the experimental data by least squares regression.

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

PC46

Developmental changes in DMT-1 mRNA expression correlate with the magnitude of Fe^{2+}-evoked electrogenic transport in human intestinal Caco-2 cells

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Exposure to iron results in the manifestation of an inward short circuit current (I_{sc}) in Caco-2 epithelia (Scott *et al.* 2002). This electrogenic pathway is consistent with DMT-1-mediated iron uptake. Tandy *et al.* (2000) have shown that DMT-1 expression changes with age in Caco-2 cells. This study investigated the relationship between developmental changes in DMT-1 mRNA expression and the magnitude of Fe^{2+}-evoked I_{sc} responses.

Caco-2 cell monolayers were grown for 7, 14, 21 or 28 days before being voltage-clamped in Ussing chambers for I_{sc} determinations. Cumulative concentration-responses were performed for iron in isotonic mannitol/HEPES buffer (37°C). Apical pH was 6.0, whilst basolateral pH was 7.4. Iron (iron-ascorbate; 1:10 molar ratio) was applied apically in concentrations ranging from 25-1000 μM. In parallel experiments, complementary DNA (cDNA) PCR primers for human DMT-1 IRE (iron regulatory element) and non-IRE were designed and first strand cDNAs were synthesised by priming with random hexamers. Reverse transcription was performed using DNase treated Caco-2 RNA at 7, 14, 21 and 28 days. Real-time PCR amplification and analysis was performed using a 7700 Sequence Detection System and SYBR Green Mastermix (Applied Biosystem). Standard curves were generated from cDNA (from total Caco-2 RNA). C_T (cycle threshold) values were used to calculate linear regression lines to determine DMT-1 mRNA levels.

After 7 and 14 days, Fe^{2+} failed to induce an I_{sc} response. However, after 21 and 28 days, the I_{sc} evoked by Fe^{2+} was concentration-dependent and saturable (day 21: K_M = 238 ± 54 (4) μM and V_{max} = 1.34 ± 0.28 (4) $\mu A.cm^{-2}$; day 28: K_M = 164 ± 20 (5) μM and V_{max} = 1.00 ± 0.08 (5) $\mu A.cm^{-2}$; mean ± SEM (n)). Caco-2 cells also demonstrated age-dependent expression of DMT-1 IRE mRNA (day 7 = 0.63 ± 0.19 (6); day 14 = 1.86 ± 0.23 (6); day 21 = 58.46 ± 20.5 (6); day 28 = 5.50 ± 1.17 (6), values expressed as DMT-1 mRNA (ng):18S mRNA (ng)). DMT-1 IRE mRNA expression was greater at day 21 than at days 7, 14 or 28 ($P<0.001$, one way ANOVA). There was no change in DMT-1 non-IRE mRNA levels.

These data demonstrated that only DMT-1 IRE mRNA expression was time-dependent in Caco-2 cells. Maximal expression occurred around 21 days in culture and coincided with the maximum electrogenic response to iron in these cells. This is consistent with iron-evoked changes in I_{sc} being due to apical Fe^{2+} uptake mediated by DMT-1.

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

PC47

Effects of hypoxia on membrane transport in articular chondrocytes

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Articular cartilage is avascular and consequently hypoxic: O_2 tensions of between 4% and 10% have been reported for the tissue (Grimshaw & Mason, 2000) and values for the deepest zones of cartilage are likely to be even lower. Routine protocols for isolating cartilage tissue necessarily involve some exposure to normoxia and, for this reason, articular cartilage is frequently studied in vitro at ambient O_2 levels (21%). However, this may be inappropriate since we have recently demonstrated that Na^+/K^+ pump activity is greater under hypoxic conditions (Fairfax et al., in press). In this report, we extend our study to the effects of more sustained, and therefore more physiological, restorations in O_2 tension to hypoxic levels on chondrocyte Na^+ and H^+ homeostasis.

Cartilage slices were taken from bovine and equine metacarpophalangeal joints of animals humanely killed (under Home Office guidelines) for other purposes. Chondrocytes, isolated overnight by collagenase digestion at ambient levels of O_2, were incubated for 2 h at 21% or nominally zero O_2 before assaying ion transport at 37°C. pH_i was determined fluorimetrically using BCECF (Wilkins & Hall, 1992). Ouabain-sensitive ^{86}Rb influx was used as a measure of Na^+/K^+ pump activity, and amiloride-sensitive $^{22}Na^+$ influx for NHE activity. Statistical significance was determined using Student's unpaired t test.

Steady state pH_i was 6.95 ± 0.06 and 6.92 ± 0.02 under normoxic and anoxic conditions respectively (mean ± S.E.M., not significant (n.s.), n = 3 for all experiments). Na^+/K^+ pump activity was 41.6 ± 7.6 and 43.1 ± 2.4 nmol (10^6 cells) h^{-1} in oxygenated and deoxygenated cells (n.s.). By contrast, amiloride (100 μM)-sensitive Na^+ influx during recovery from acid load was 72.4 ± 3.9 nmol (10^6 cells) h^{-1} in normoxia and 42.6 ± 10.0 in anoxia ($p < 0.05$). Na^+ dependent acid equivalent efflux, measured fluorimetrically, decreased from 2.13 ± 0.12 in (normoxia) to 1.28 ± 0.28 mM min^{-1} (anoxia; $p < 0.05$). Finally, bafilomycin (200nM)-sensitive acid efflux increased from 0.36 ± 0.40 to 0.91 ± 0.69 mM min^{-1} (n.s.).

The present results show that, despite the fall in glycolysis (the negative Pasteur effect; Lee & Urban, 1997), in longer term hypoxia Na^+/K^+ pump activity is protected. By contrast, NHE activity was halved. These findings have implications for ion homeostasis in articular chondrocytes in vivo and for the use of chondrocytes isolated at ambient O_2 tensions.

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

PC48

Role of basolateral K^+ channels in ACh-induced anion secretion in porcine tracheal epithelia

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ACh induces anion secretion in intact porcine tracheal epithelia (Constable *et al* 2003) and isolated submucosal glands (Yang *et al* 1988) but the mechanism of activation is unclear. ACh and the influx of Ca^{2+} resulted in Cl^- secretion across the apical membrane of isolated porcine tracheal submucosal glands (Yang *et al* 1988). ACh may therefore act to increase $[Ca^{2+}]_i$, activating basolateral Ca^{2+}-activated K^+ (K_{Ca}) channels, thus providing an electrical driving force for Cl^- exit across the apical membrane. Basolateral K^+ channels have been demonstrated in several respiratory epithelia and have been shown to be involved in anion secretion (McCann and Welsh 1990).

Here we investigate the role of basolateral K^+ channels in the ACh-induced anion secretion in porcine tracheal epithelium.

Tracheas were removed from humanely killed pigs and the epithelial layer dissected from underlying cartilage. Epithelial sheets were mounted on Ussing chambers and bathed in HCO_3^- buffered solution, gassed with 95%O2/5%CO2 at 37°C.

ACh evoked a peak increase in I_{sc} (17.6±1.3μAcm^{-2}, mean ± S.E.M., n=6) that slowly decayed over time. In the presence of the non-specific K^+ channel inhibitor barium the response to ACh was significantly inhibited (40.4±9.1%, n=6, student's paired t-test used throughout, p=0.05 taken as threshold of significance), indicating a role for K^+ channels in this process. Specific mediators of different K^+ channels were used to identify which types are involved in the ACh-induced anion secretion. Chromanol 293B, an inhibitor of voltage-gated K^+ channels had no effect on the response to ACh, suggesting if present, these channels do not play a role in this process. In contrast the K_{Ca} channel inhibitor clotrimazole significantly inhibited the response to ACh (42.1±7.9%, n=6), indicating a role for K_{Ca} channels in the ACh-induced anion secretion. In support of this 1-EBIO, a K_{Ca} channel activator, augments the anion secretion evoked by ACh (63.6±27.1%, n=6).

Together these data suggest ACh evokes anion secretion by activating basolateral K_{Ca} channels, presumably by increasing $[Ca^{2+}]_i$ thus driving Cl^- secretion across the apical membrane via anion channels.

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PC49

Effect of forskolin on acid and base secretion by isolated distal bronchi

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Secretion of HCO_3^- by airway submucosal glands is essential for normal liquid and mucus secretion (2). Since the liquid bathing the airway surface is acidic, we proposed that the surface epithelium may acidify HCO_3^--rich glandular fluid (1). We showed that isolated distal bronchi, containing both glandular and surface epithelium, can both acidify and alkalinise their lumens (2). The aim of this study was to investigate the effect of forskolin on acid and base equivalent secretion in these airways.
Porcine distal bronchi were isolated from pigs humanely killed with an overdose of sodium pentobarbital, cannulated in a bath containing HCO_3^--buffered solution and perfused with similar solution, in which NaCl replaced $NaHCO_3$. This solution was lightly buffered (0.6 mM pH unit^{-1}) with KH_2PO_4 and NaOH to pH7, 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.
As previously shown (2), upon perfusion through the airway lumen pH of the circulating solution (pH_{Lumen}) initially fell, by 0.055 ± 0.005 pH units (means ±S.E.M., n = 7). Pretreatment (3min) with forskolin (10μM) had no effect on this acidification (0.061 ± 0.008 pH units, P > 0.05, Student's paired t test used throughout). After this initial acidification pH_{Lumen} became relatively stable with a trend to alkalinisation (3). Addition of forskolin at this stage had no effect on pH_{Lumen} (basal rate of alkali secretion 0.34 ± 0.13μmoles hr^{-1}, after forskolin addition 0.36 ± 0.11μmoles hr^{-1}, n=9, P > 0.05). In contrast the glandular secretagogue acetylcholine (ACh) stimulates alkalinisation (2). Addition of bumetanide, to block Cl^- secretion, stimulates HCO_3^- secretion in these airways and augments the response to ACh (2). Bumetanide (100μM), however, did not alter the response to forskolin (pre-forskolin alkalinisation 0.57 ± 0.11μmoles hr^{-1}, after forskolin 0.59 ± 0.02μmoles hr^{-1}, n=4, P > 0.05). These results suggest that increases in intracellular cAMP do not control the secretion of acid and base equivalents in these airways. To further investigate these findings, the effect of forskolin on the ACh-evoked secretion of HCO_3^- was studied. As previously described (3), ACh stimulated secretion of HCO_3^- (0.36 ±0.05 μmol hr^{-1}). This was significantly inhibited (34 ± 12 %, n=7, P<0.05)) by prior treatment with forskolin. Thus, whilst cAMP-mediated intracellular signalling pathways may not themselves control acid and base secretion in these airways, they seem to modify the effect of ACh on HCO_3^- secretion.

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

PC50

Forskolin induces SGLT1 trafficking in chicken jejunum

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Forskolin (FSK) is an activator of the cAMP signalling cascade that increases D-glucose (D-Glc) transport across the brush-border membrane of the enterocyte (Moreto *et al.* 1984; Sharp & Debnam, 1994). We have now studied the cellular mechanisms involved in FSK-stimulation of apical D-Glc uptake.
The experiments were carried out using 4- to 6-week-old chickens. The everted jejunum was incubated for 20 min at 25°C in a medium with or without FSK (50 μM), and in the presence of the trafficking inhibitors wortmannin (80 μM; WORT) and brefeldin A (0.25 mM; BFA), or the combination of FSK plus WORT or FSK plus BFA. In parallel experiments, the jejunum was also incubated with a combination of FSK plus the unspecific protein kinase inhibitor staurosporine (1 μM; STA) or the protein kinase A inhibitor H8 (60 μM). After incubation, brush-border membrane vesicles (BBMV) were prepared from mucosal scrapings and used for determination of the kinetic constants of D-Glc uptake, and specific phlorizin and cytochalasin B binding to BBMV, as described previously (Garriga *et al.* 1999). Experiments were approved by the Ethical Committee of the University of Barcelona.
Table 1 shows that FSK strongly stimulated maximal apical D-Glc transport rate (V_{max}). In contrast, incubation with WORT and BFA reduced D-Glc V_{max} by 30% and 25%, respectively. When vesicles were incubated with FSK together with WORT or BFA, no stimulation of D-Glc V_{max} was observed. Addition of inhibitors of vesicle trafficking prevented FSK-induced D-Glc V_{max} stimulation, and the same results were found using protein kinase inhibitors. No effects were observed on Michaelis or diffusion constants in any experimental condition. The absence of specific cytochalasin B binding to BBMV indicated that no GLUT2 was present in BBMV. Specific phlorizin binding measurements demonstrated that the changes in D-Glc V_{max} were due to the changes in the number of SGLT1 present in BBMV.
We conclude that in the chicken jejunum stimulation of D-Glc uptake by FSK is mediated by changes in SGLT1 trafficking from cytoplasmatic compartments to the brush-border membrane. Results also indicate that activation of protein kinase A is involved in this regulatory mechanism.

Table I. Kinetic constants of D-Glc transport across BBMV

	V_{max}	K_m	K_d
	(pmol D-Glc(mg prot)$^{-1}$ s)$^{-1}$)	(mM)	(nL(mg prot)$^{-1}$ s^{-1})
Control	139 ± 11	1.30 ± 0.24	10.6 ± 1.8
FSK	220 ± 17a	1.25 ± 0.40	11.3 ± 2.0
WORT	97 ± 6b	1.55 ± 0.34	9.6 ± 1.9
BFA	104 ± 10b	1.37 ± 0.29	10.4 ± 0.8
FSK + WORT	136 ± 15	1.31 ± 0.19	9.9 ± 1.0
FSK + BFA	141 ± 10	1.60 ± 0.35	11.0 ± 1.0
FSK + STA	148 ± 18	1.58 ± 0.34	12.4 ± 1.4
FSK + H8	137 ± 11	1.38 ± 0.11	10.3 ± 1.3

Values are means ± S.E.M. of 3 different vesicle preparations. Values with different superscripts were significantly different (ANOVA; P < 0.05).

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

PC51

The pair vitamin C-glutathione in the defense against oxidative stress in human endothelial cells

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Vitamin C and glutathione are important antioxidants that are present at millimolar concentrations in most cells and have a central role in antioxidant cellular defenses. All cells have the capacity to synthesise glutathione, while vitamin C is obtained from extracellular sources and is transported intracellularly. In vitro studies have revealed that glutathione participates in the accumulation and the maintenance of constant intracellular levels of reduced vitamin C. In vivo studies have revealed that the oxidative damage generated by glutathione deficiency is prevented by the administration of vitamin C, and the negative effects asociated with vitamin C deficiency are delayed by the administration of glutathione esters, thus indicating a two-way functional relationship between these two antioxidants. Using as models primary cultures of human umbilical vein and tonsil endothelial cells, and the endothelial cell line ECV-304, we determined the content and the role of glutathione in the cellular acumulation of vitamin C, and examined the role of vitamin C, gutathione, and the pair vitamin C-glutathione in the resistance of the endothelial cells to acute treatment with hydrogen peroxide. Although glutathione was present in the endothelial cells at similar concentrations (2-3 mM, mean ± S.E.M., n=6), the accumulation of vitamin C was glutathione independent in the tonsil endothelial cells. Acute treatment with hydrogen peroxide caused a massive decrease in intracellular glutathione, decrease which was related to the relative resistance of the cells to the treatment. Thus, cells lacking glutathion after treatment with L-buthionide(SR)sulfoximine and diethylmaleate showed a much increased sensitivity to oxidant-induced cell death, and glutathione depletion affected also the capacity of the cells to proliferate. Cells containing graded concentrations of glutathione showed a concentration-dependent increase in their resistance to oxidative stress, and a similar effect was observed in cells containing graded concentrations of ascorbic acid. However, vitamin C and glutathione were clearly different in their respective capacitie to protect cells from oxidative stress. Full protection against hydrogen peroxide treatment was observed in endothelial cells containing vitamin C concentrations as low as 0.2 mM (n=6); in contrast, at least 2 mM (n=4) glutathione was required to observe a similar protective effect. Our data are compatible with the concept that vitamin C and glutathione play a complementary role in the defense of the endothelial cells to oxidative stress, depending on the level of the oxidant insult.

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PC51A

Altered basolateral K^+ channel activity as the basis for epithelial cell depolarisation in ulcerative colitis

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A decrease in the colon's ability to absorb Na^+ and water is a major factor in the etiology of diarrhea in ulcerative colitis (UC). Vectorial ion transport in general is influenced by cell membrane voltage, which in turn depends upon K^+ channel activity. Since colonic epithelial cells are depolarized in UC (Sandle *et al.* 1990), we used patch clamp recording to compare the activity of small (SK;~5pS), intermediate (IK; ~30pS), and large (BK; ~130pS) conductance K^+ channels in the basolateral membranes of isolated human colonic crypts from control and UC patients.
With informed consent, intact crypts were isolated (Sandle *et al.* 1994) from 4-5 rectosigmoid mucosal biopsies taken from 28 patients undergoing colonoscopic evaluation of functional bowel symptoms (controls), and 7 patients with moderately active UC. Cell-attached patches were obtained on the basolateral membrane of cells in the mid-third of the crypts, with NaCl Ringer solution in the bath and KCl Ringer solution in the pipette. Data are expressed as mean ± S.E.M., Student's t test was used to analyse statistical significance ($P < 0.05$).
In control patients, 37% of patches contained SK channels, 63% contained IK channels, and 5% contained BK channels. By contrast, in UC patients, 70% of patches contained SK channels, 43% contained IK channels, and BK channels were absent. Furthermore, SK channel activity (NPo, where N = number of channels in the patch, and Po = single channel open probability) was significantly greater in UC patients (0.93 ± 0.19) than in control patients (0.11 ± 0.02, P<0.001), whereas IK channel activity was significantly less in UC patients (0.31 ± 0.08) than in control patients (0.68 ± 0.08, P<0.05), and the low level of BK channel activity in controls (0.01 ± 0.01) disappeared in UC. Assuming K^+ channels to be uniformly distributed within the basolateral membrane, overall basolateral membrane K^+ conductance in UC patients (46.6%, 53.4% and 0% of which was related to SK, IK, and BK, respectively) was 63% (P<0.02) lower than control patients (2.4%, 89.2%, and 8.4% of which was related to SK, IK, and BK, respectively).
UC is associated with substantial shifts in the pattern of basolateral membrane K^+ channel expression/activity in colonic crypt cells. This decreases overall basolateral K^+ conductance by 63%, which provides an explanation for the epithelial cell depolarization that underlies defective Na^+ absorption in this disease.

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