C1

Evidence of apical to basal organisation of microtubules in the mpkCCDc14 mouse collecting duct cell line

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Microtubules (MT) are involved in the delivery of secretory vesicles, including those carrying AQP2 to the apical surface of renal collecting duct principal cells. MT are polar structures, which typically have their minus end anchored in an organising centre (MTOC), while their plus ends grow away into the cell. Different motor proteins are involved in vesicle transport towards the plus and minus ends of MT, so which motors are required for movement towards a particular cell membrane will depend on MT organisation within the cell. In fibroblastic and neuronal cells, the MTOC is perinuclear, with the plus ends of microtubules being near the plasma membrane. In contrast, there is some evidence that in epithelial cells MT are nucleated from a diffuse apical network, and project towards the basal pole of the cell. To investigate this, we have immunolabelled microtubules and AQP2 in the collecting duct cell line mpkCCDc14 (grown on glass coverslips and filters), before, during, and after treatment with 33 μM nocodazole and/or 1 nM dDAVP. Cells were photographed on a Zeiss LSM 510 confocal microscope, and images analysed using ImageJ software. In control cells, there was an abundant apical network of MT, and a sparser basal network. In between, there appeared to be bundles of MT running around the nucleus. Nocodazole treatment (30 min) decreased MT labelling by 95± 2% in the apical two thirds of the cells ($p<0.05$, $n=10$), although a few wavy microtubules could still be seen. After 40 minutes washout, microtubules had started to reappear, predominantly in the form of an aster in the apical pole of the cells. dDAVP treatment was able to increase the fraction of AQP2 in the apical third of the cell from 30% to 50% of the total ($p<0.05$, $n=10$): this redistribution was prevented by nocodazole treatment. These results are consistent with the hypothesis that MT in these cells are organised with their "organising centres" (and thus their minus ends) in the apical part of the cell, and their plus ends projecting down into the cell. This is consistent with our previous evidence that dynein (a minus-end directed motor) associates with AQP2-containing vesicles, and is likely to be important in AQP2 shuttling in response to vasopressin (Marples *et al*, 1998).

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We thank Dr. M. Peckham for advice and antibodies.

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

C2

Stretch-activated channels in the postnatal rat pulmonary epithelium

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The liquid in the lumen of postnatal lung is in a homeostatic state: there is a balance between absorption and secretion, this maintains the lining layer at a mean depth of ~0.15μm (1). The instillation of liquid into the postnatal lungs result in a shift of the balance and net absorption of liquid predominates. The absorption is the result of active sodium movement, across the pulmonary epithelium and the movement of water down its osmotic gradient (2).

In a series of experiments, to examine the effect of luminal volume on liquid absorption, adult Wistar rats were anaesthetised using a 1:1:2 mixture of midazolam, fentanyl citrate/fluanisone and water (2.7 $ml.kg^{-1}$ip). The animals were ventilated with room air and pulmonary artery and left atrium were catheterised. The lungs were perfused with a modified Ringer's solution containing 3% albumin. A known volume of liquid, containing an impermeant tracer (Blue D) was instilled into the lungs. The volumes of lung liquid (LL) used were: 10, 15 or 30 $ml.kg^{-1}$. Blue D concentration was determined from the absorbance of samples of LL at 620nm and this was used to calculate the change in LL volume with time (J_v).

J_v during the first 40 minutes of sampling was the same for all volumes of LL used. During the following 40 minutes, J_v increased in the 30 $ml.kg^{-1}$ experiments but remained the same for the other 2 volumes (Table 1). The increase did not involve either the epithelial sodium channel (ENaC) or the cyclic nucleotide gated channel (CNG) as blockers of both channels, amiloride (50μM) and l-cis-diltiazem (100μM) respectively, had no effect on the increase in J_v. In contrast, gadolinium chloride ($GdCl_3$; 10μM) blocked the increase in J_v (Table 1) suggesting the involvement of stretch-activated channels (SAC).

We conclude that the permeability of the postnatal rat pulmonary epithelium is unlikely to have been altered by LL volumes up to 30 $ml.kg^{-1}$ and that recruitment of SAC is one mechanism of maintaining homeostatic control. Fisher and Margulies (3) have shown that Na^+- K^+-ATPase activity increases with cyclic but not tonic stretch of alveolar type 2 cells in culture. We cannot discount this mechanism in the whole lung but as $GdCl_3$ prevented the increase in J_v seen at 30 $ml.kg^{-1}$, it is likely that this effect is mediated by SAC in the epithelium.

J_v for 2 consecutive sampling periods using 10, 15 or 30 $ml.kg^{-1}$ body weight LL volume; also J_v at 30 $ml.kg^{-1}$ when $GdCl_3$ was instilled into the LL at 30 mins.

	10 $ml.kg^{-1}$	15 $ml.kg^{-1}$	30 $ml.kg^{-1}$	30 $ml.kg^{-1}$ & 10 μM $GdCl_3$
40 - 80 mins	-0.81 ± 0.07	-0.87 ± 0.08	-0.99 ± 0.11	-0.71 ± 0.18
90 - 130 mins	-0.77 ± 0.10	-0.88 ± 0.08	-1.49 ± 0.22*	-0.79 ± 0.21

Results are mean J_v ± SEM ($ml.hr^{-1}.kg^{-1}$ body weight). * $P = 0.023$ compared to 10 $ml.kg^{-1}$ and $P = 0.014$ compared to 15 $ml.kg^{-1}$ using unpaired t-test.

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

C3

Calcium-dependent secretion is differentialy affected by genetic background in colon of different mouse strains

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The mouse is the model of choice in studies of physiology and pathophysiology of intestinal electrolyte transport. We have observed differences in electrical tissue properties and responses to agonists that induce electrogenic secretion of electrolytes among mice strains, that are important enough to preclude comparisons between strains and might be crucial when choosing the genetic background for knockout of genes of interest. We have investigated some of these differences by electrophysiological methods.

A piece of distal colon of B6, Black Swiss or 129 Sv mice was placed in Ussing chambers in a bicarbonate buffered solution, gassed with 95% O_2/5% CO_2 at 37^s C. The transepitheial potential difference was monitored continuously and 5 μA pulses were passed to calculate tissue resistance and equivalent short circuit current (Isc). To induce cAMP-mediated Cl^- secretion 100 μM IBMX and 1 μM forskolin were added. Bumetanide (100 μM) was added to the basolateral side of the tissue to block NKCC1 co-transporter. Apical Ba^{2+} (5 mM) inhibited K^+ channels. Basolateral carbachol (100 μM) or Histamine (150 μM) were used as Ca^{2+} agonists. 4-DAMP, Pirenzepine and Mepyremine are M3, M1 and H1 receptors blockers respectively. Animals were killed under IUCAC regulations and approval of the ethical committee.

We observe that CCH induced the largest anionic secretory response in B6 tissue, compared to that of Black Swiss and 129Sv colon (-200, -163 and -133 μA cm^{-2} respectively). The use of blockers for muscarinic and histaminergic repector indicated that M3 and H1 receptors were involved in the secretory responses in all types of colon. Histamine was able to induce K^+ and Cl^- secretory responses in the B6 colon while in the other two only K^+ secretion was observed. By adding Ba^{2+} to the mucosal side of the chamber a K^+ secretory process was blocked, and shown to be larger in the 129 Sv colon (15 ± 1.2 μA cm^{-2}) compared to B6 and Black Swiss (both around 10 μA cm^{-2}). Bumetanide inhibits K^+ secretion in Sv 129 colon only. In all strains K^+ secretion was activated by cAMP. RT-PCR studies demonstrated the presence of 2 KCNMA1 splice variants in colon: ZERO and STREX.

We conclude that the observed differences in calcium-activated K^+ and Cl^- secretion are not due to the presence of different receptors among strains. The cAMP dependence of K^+ secretion suggests that the ZERO variant of KCNMA1 is active in colon, rather than STREX that is inhibited by cAMP. A difference in the number of channels involved in these processes cannot be discarded as we have previously observed that the calcium-activated Cl^- secretion is dependent on the expression level of basolateral KCNN4 channel rather than apical CFTR channels. Our results also show the importance of the genetic background in determining intestinal transport properties.

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C4

Iodothyronine interactions with System L1 amino acid transport in mouse 3T3-L1 adipocytes

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White adipocytes are an important target tissue for thyroid hormones, which enter the cells largely by a System L1-type amino acid transporter *en route* to exerting genomic actions in the nucleus (Ritchie et al., 2001). We are investigating the regulation of this process using differentiated 3T3-L1 adipocytes and L-[^{3}H]phenylalanine as a transportable tracer for System L1 activity. 3T3-L1 cells were cultured at 37^{o}C, 5% CO_2 in Dulbecco's Modified Eagles Medium (high-glucose) with 10% serum and 1% antibiotic/antimycotic solution; they were differentiated into adipocytes using 1 μg.ml^{-1} insulin, 5 μM isobutylmethylxanthine and 100 pM dexamethasone (Hyde et al., 2001) .

Phenylalanine uptake into 3T3-L1 cells was saturable (K_m of 31 μM, V_{max} of 2.6 ± 0.5 nmol. mg $protein^{-1}$. min^{-1}; mean ± sem, n=6) and inhibited by the synthetic System L substrate 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH):- 5 μM phenylalanine uptake (0.465 ± 0.065 nmol. mg $protein^{-1}$. min^{-1}, n=12) was reduced by 98% in presence of 10 mM BCH. Phenylalanine uptake was competitively inhibited by T_3 (K_i of 1.2 μM) but not by Triac (a T_3 derivative lacking the α-amino grouping) and was also blocked by leucine and rT_3 in a manner consistent with expected substrate interactions for the System L1 transporter (Ritchie et al., 1999; Friesema et al., 2001). We detected mRNA for LAT1 (the catalytic subunit of System L1) in 3T3-L1 cells using RT-PCR. Efflux of preloaded L-[^{3}H]phenylalanine from 3T3-L1 adipocytes was *trans*-stimulated by external leucine (up to 20-times stimulation of basal efflux), demonstrating the obligatory exchange mechanism of System L1 transport (Meier et al., 2002). T_3 has a relatively low V_{max} for transport by System L (Ritchie et al., 1999) and was unable to significantly *trans*-stimulate L-[^{3}H]phenylalanine efflux at 10 μM (a concentration approaching the limit of T_3 solubility in culture medium), although it did inhibit the *trans*-stimulatory effect of 10 μM leucine. System L transport activity was reduced by 40% in 3T3-L1 cells cultured in thyroid-hormone depleted (charcoal-treated) medium for 24 hours.

The present results confirm a strong competitive interaction between iodothyronines (T_3, rT_3) and amino acids for transport by System L1 in adipocytes. Down-regulation of iodothyronine uptake by adipocytes in the hypothyroid state, as also reported previously (Ritchie et al., 2001), should reduce their catabolism and help conserve hormone and iodine availability to other tissues.

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C5

Slc26 anion transporters involvement in acid-activated duodenal HCO_3^- secretion

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Background: HCO_3^- secretion protects the proximal duodenum against damage by gastric acid. Duodenocytes express CFTR as well as at least three members of the SLC26 family (Slc26a3, 6 and 9) in the apical membrane. **Aim and Methods:** To establish the importance of the different apical anion transporters during basal, acid- and forskolin (FSK)- stimulated duodenal HCO_3^- secretion *in vivo*. Mice were anesthetized by a spontaneous inhalation of isoflurane (Forene, Abbott Scandinavia, Kista, Sweden). The inhalation gas contained a mixture of ~20–30% oxygen, ~70–80% air, and 2.0 +/- 0.2% isoflurane with the use of an isoflurane pump (Univentor 400 Anaesthesia Unit, AgnTho, Lidingo[|#168#|], Sweden) and was administered continuously through a breathing mask. The depth of the anaesthesia was tested by probing the pedal reflex. CFTR-, Scl26a3, 6 and 9-deficient mice and WT litter-mates were anesthetized, the proximal duodenum was luminally perfused with saline, 10^{-4} M FSK, or pH 2.2 for 5 min, and HCO_3^- was continuously titrated in the perfusate during controlled systemic acid/base parameters. The relative crypt and villous expression of the trans-porters was measured by immunohistochemistry, laser dissection and qPCR. **Results:** Basal duodenal HCO_3^- secretion was slightly reduced in the *Slc26a6*$^{-/-}$, and more strongly reduced in the *Slc26a3*$^{-/-}$, *Slc26a9*$^{-/-}$, and in CFTR-deficient mice compared to WT littermates. FSK-stimulated secretory response was normal in *Slc26a3*$^{-/-}$, *Slc26a6*$^{-/-}$, *Slc26a9*$^{-/-}$ mice, and was virtually abolished in CFTR-deficient mice. Surprisingly, acid-activated HCO_3^- secretion was unaltered in the *Slc26a6*$^{-/-}$ duodenum, but strongly reduced in *Slc26a3*$^{-/-}$ and *Slc26a9*$^{-/-}$ duodenum, and abolished in the CFTR-deficient duodenum. Laser dissection and immunohistochemistry revealed a villous-predominant expression of Slc26a6, Slc26a3 and NBCn1 and a crypt predominant expression of CFTR and Slc26a9. **Conclusions:** Genetic deletion of intestinal Slc26 anion transporters reveals their differential involvement in basal, acid-stimulated and FSK-stimulated HCO_3^- secretion in murine duodenum. Most likely, different signalling in acid- vs FSK-stimulated HCO_3^- secretion explains this differential involvement, rather than the differential crypt-villus expression pattern.

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C6

Glucocorticoid-inducible leucine zipper protein 1 (GILZ1) induces membrane Na^+ current (I_{Na}) in hormone-deprived airway epithelial cells

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Glucocorticoids can activate a Na^+-permeable conductance in H441 airway epithelial cells via a mechanism dependent upon phosphoinositide-3-kinase (PI3K) (Brown *et al.*, 2008; Inglis *et al.*, 2009). It is now well established that PI3K controls the activity of serum and glucocorticoid-inducible kinase 1 (SGK1) (Kobayashi and Cohen, 1999), and this regulatory kinase controls over the abundance of epithelial Na^+ channels in the plasma membrane. The fact that the transient expression of a constitutively active form of SGK1 (SGK1-S422D) mimics the effects of glucocorticoid stimulation by inducing Na^+ current (I_{Na}) in hormone-deprived cells therefore suggests that SGK1 may mediate the physiological effect of glucocorticoid stimulation (Brown *et al*. 2008). However, not all data are consistent with this hypothesis since we have also shown that activating PI3K, either by insulin stimulation or by the transient expression of a membrane-anchored form of the catalytic PI3K-P110α subunit (CD2-P110α, see Reif et al, 1996) does not alter the electrical properties of hormone-deprived cells. Moreover, we have recently shown that the expression of CD2-P110α does increase cellular SGK1 activity (N. McTacvish and SM Wilson, unpublished) suggests strongly that factors other than SGK1 must contribute to the glucocorticoid-dependent control of I_{Na} in these cells. It is therefore interesting that studies of renal epithelial cells have shown the glucocorticoid-inducible leucine zipper proteins (GILZ1-3) contribute to the control of I_{Na} by (i) supressing signalling via extracellular signal dependent kinases (ERK1/2) and (ii) by facilitating the effects of SGK1 upon the abundance of ENaC in the plasma membrane (Soundararajan et al., 2009). The present study therefore explored the effects of GILZ1 upon the electrical properties of hormone-deprived H441 cells.

The first such studies confirmed that glucocorticoid-deprived H441 cells do not normally display any I_{Na} and showed that dexamethasone consistently induced ~60 pA $cell^{-1}$ of current (Fig 1A). Although this response is dependent upon PI3K (Inglis *et al.* 2009), increasing cellular PI3K activity by transient expression of CD2-P110α did not reproduce this physiological response to dexamethasone stimulation (Fig 1B). However, transient expression of GILZ1 consistently induced I_{Na} indicating that this endogenous protein can fully reproduce the electrophysiological consequences of glucocorticoid stimulation. These data therefore suggest that GILZ1 may contribute to the hormonal control of Na<up>+ transport in human airway epithelia.

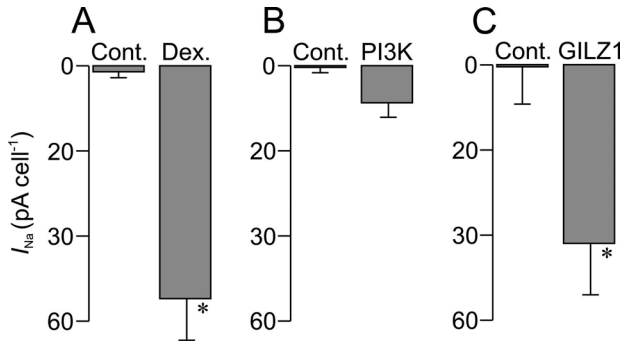

Figure 1. Membrane currents were recorded from single H441 cells using the perforated patch configuration of the whole cell recording technique. Currents were initially recorded under quasi-physiological ionic gradients, and the measurement repeated 20 - 30 s after $[Na^+]_o$ had been lowered to 10 mM ($NMDG^+$ substitution). The $[Na^+]_o$-dependent component of the current flowing at -82 mV (I_{Na}) was then quantified and presented as mean ± standard error (n > 5 in all experiments). Unless otherwise stated all cells were maintained in dexamethasone-free medium for 24 – 36 h before being used in experiments. A. Effects of dexamethasone-stimulation (0.2 μM, 24 – 36 h). B Effects of increasing cellular PI3K activity by transiently expressing CD2-P110α. (B) Effects of transiently expressing GILZ1. Control cells in B and C were transfected with empty vector.

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C7

Epilepsy, ataxia, sensorineural deafness, tubulopathy (EAST) syndrome – KCNJ10 involved in renal salt and Mg^{2+} handling

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Introduction and Methods

Five patients from two consanguineous families presented with epilepsy from infancy, severe ataxia, moderate sensorineural deafness, and a renal salt losing tubulopathy with normotensive hypokalemic metabolic alkalosis. We investigated the genetic basis of this new autosomal recessive disease termed EAST syndrome.

Whole genome linkage analysis was performed in a family with four affected children and multiple consanguineous links. Newly identified mutations in the Kcnj10 potassium channel gene were evaluated using Xenopus oocytes. Localization and function were characterised in WT and KCNJ10 KO mice.

Results

Linkage analysis identified a single relevant locus on chromosome 1q23.2 with a LOD score of 4.98. This region contained Kcnj10, which encodes a potassium channel, also known as Kir 4.1, expressed in brain, inner ear, and the kidney. Sequencing of this gene revealed homozygous missense mutations in affected persons in both families. Mutant KCNJ10 showed significantly decreased potassium currents in Xenopus oocytes (R65P decreased to about 25%, while currents from G77R were not significantly different from water-injected oocytes, N>3, n>20). Kcnj10 KO mice became dehydrated and exhibited evidence of renal salt wasting. Immunohistochemistry revealed prominent expression in the distal tubule (n=3), explaining salt loss and changes in renal handling of divalent cations. Expression was confined to the basolateral membrane, where KCNJ10 may function in both generating the driving force for Cl- reabsorption through ClC-K/barttin channels and supplying K+ to the Na+/K+-ATPase, both affecting NaCl reabsorption through NCC.

Discussion

In conclusion, mutations in KCNJ10 cause epilepsy, ataxia, sensorineural deafness, and tubulopathy. Our findings indicate a major role for KCNJ10 in hearing, regulating excitability in the brain, renal salt handling and possibly blood pressure maintenance and its regulation in humans.

A. Zdebik wishes to express his gratitude towards Thomas Jentsch for help with setting up the new laboratory in London

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C8

Functional characterization of mutations of the epithelial sodium channel (ENaC) associated with atypical cystic fibrosis

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Cystic fibrosis (CF) is an autosomal recessive hereditary disease usually caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. A functional interaction of the epithelial sodium channel (ENaC) and the CFTR chloride channel is suspected to play a role in the pulmonary pathophysiology of cystic fibrosis (1). Transgenic mice overexpressing the β-subunit of ENaC in the airways develop a CF-like pulmonary phenotype (2). Interestingly, CF-like pulmonary symptoms have also been reported in patients with pseudohypoaldosteronism type1 (PHA1) caused by loss-of-function mutations in ENaC (3). Thus, increased or decreased ENaC activity in respiratory epithelia may be associated with pulmonary symptoms (4). Recently, several ENaC mutations were identified in patients with atypical CF in whom a mutation could not be identified on both CFTR alleles (5). Interestingly, these mutations include a gain-of-function (W493R) and a loss-of-function mutation (F61L) in the α-subunit of ENaC. To investigate the functional effect of these mutations, we co-expressed the

mutant α-subunits together with wild-type β- and γ-subunits in *Xenopus laevis* oocytes and compared the two mutant channels ($\alpha_{W493R}\beta\gamma$ or $\alpha_{F61L}\beta\gamma$) with wild-type αβγ-ENaC. The W493R mutation stimulated ENaC-mediated whole-cell currents by about fourfold without a concomitant increase in single-channel conductance or channel surface expression. The αF61L mutation reduced ENaC mediated whole-cell currents by about 90%, decreased channel surface expression by about 40%, and did not alter the single-channel conductance. These findings indicate that the mutations mainly affect the average open probability *(P_O)* of ENaC. This was confirmed by experiments using the βS520C mutant ENaC which can be converted to a channel with a P_O of nearly one, and by experiments using chymotrypsin to proteolytically activate the channel. Interestingly, the ENaC-activator S3969 partially rescued the loss-of-function phenotype of the F61L mutation. We conclude that gain-of-function and loss-of-function mutations may contribute to respiratory symptoms in atypical CF patients and that a mutant channel with a partial loss of function may be amenable to treatment with an ENaC activator.

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C9

Role of CFTR in bicarbonate secretion by Calu-3 airway epithelial cells

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The Calu-3 cell line, derived from human airway submucosal glands, expresses high levels of CFTR and is a good model for investigating Cl^- and HCO_3^- secretion in an epithelium that is adversely affected by cystic fibrosis (Hug et al., 2003). The aim of these studies was to determine whether HCO_3^- secretion across the apical membrane is mediated mainly by CFTR or by a Cl^-/HCO_3^- exchanger of the SLC26 family, as is the case in several other epithelia. Calu-3 cells were grown as polarised monolayers on Transwell polyester filters (Corning), superfused bilaterally with HCO_3^--buffered solutions and maintained at 37∞C on the stage of an inverted fluorescence microscope. Intracellular pH (pH_i) was measured with BCECF using standard microfluorometric techniques. Substitution of Cl^- with gluconate in the apical bath led to a marked alkalinization (to pH 7.83 ± 0.14, mean ± s.e.m., n=13) in 10 μM forskolin-stimulated Calu-3 cells, suggestive of Cl^-/HCO_3^- exchange. Unexpectedly, the alkalinization and subsequent recovery of pH_i were unaffected by the Cl^-/HCO_3^- exchanger inhibitor DIDS (100 μM) but were markedly inhibited by the CFTR channel blocker $CFTR_{inh}$-172 (5 μM). This suggested that the alkalinization might be due to HCO_3^- entry through the CFTR channel rather than via a Cl^-/HCO_3^- exchanger. In support of this interpretation, quantitative RT-PCR measurements indicated only low levels of SLC26 exchanger expression in Calu-3 cells (A3 <0.1%, A4 <0.1%, A6 10.4 ± 3.3% relative to CFTR, n=6) while expression of the basolateral anion exchanger AE2 was much greater (184 ± 36% relative to CFTR). However, for pH_i to rise to 7.8 through electrodiffusive entry of HCO_3^- via CFTR, the membrane potential would have to depolarize and reverse to a value of approximately +30 mV as a result of Cl^- efflux. Perforated patch-clamp recordings using gramicidin D confirmed that such large changes in membrane potential (to +27.5 ± 5.0 mV, n=5) did indeed occur in forskolin-stimulated Calu-3 cells exposed to zero extracellular Cl^-. We therefore conclude that, unlike other HCO_3^--secreting epithelia, such as the duodenum and pancreatic duct, Calu-3 airway cells secrete HCO_3^- predominantly via CFTR rather than an SLC26 anion exchanger.

Hug MJ et al. (2003). News Physiol Sci 18, 38-42

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C10

Cystic fibrosis transmembrane conductance regulator regulates an apical chloride-bicarbonate exchanger in airway submucosal gland epithelial cells

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In cystic fibrosis (CF) mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel leads to reduced airway Cl^- and HCO_3^- -dependent fluid secretion which impairs airway defence mechanisms and predisposes the lungs to bacterial infection (1). CFTR is regarded as the sole mediator of apical Cl^- and HCO_3^- secretion in airway epithelial cells. However, the discovery of SLC26 Cl^-/HCO_3^- anion exchange (AE) activity linked to the expression of CFTR in tracheal and pancreatic duct epithelial cells (2,3) as well as the demonstration of a functional interaction between SLC26 exchangers and CFTR (4), has led us to re-evaulate this hypothesis. The aim of this work was to investigate the role of SLC26 exchangers in HCO_3^- and fluid secretion from human airway cells. AE activity was assessed by real time measurements of intracellular pH (pH_i), using the pH-sensitive dye BCECF-AM, from Calu-3 cells grown as monolayers on semi-permeable supports (3). Calu-3 cells are used as a model of the serous cells of human airway submucosal glands and display CFTR-dependent HCO_3^- secretion (5). Under resting conditions apical Cl^- removal had no effect on pH_i, but caused an alkalinisation of 0.60±0.07 (mean ± s.e.m.;P<0.001 paired t-test, n=8) after 5 mins of 5μM

forskolin stimulation. A similar alkalinisation was observed following expoure to 10μM adenosine (0.53±0.02, n=4) and 300 nM VIP (0.60±0.04, n=7). The apical AE activity was insensitive to H2DIDs, but blocked by the CFTR inhibitor GlyH-101 (10μM) and the PKA inhibitor H-89 (50μM). AE activity was also reduced by 50 % in Calu-3 cells where CFTR content was knocked down by 95%. The re-acidification in pH_i following restoration of apical Cl^- was mimicked by other monovalent anions including iodide, bromide, nitrate, formate and thiocyanate. However, apical AE activity was not supported by divalent anions such as oxalate or sulphate. The profile of this apical Cl^-/HCO_3^- exchanger is consistent with Pendrin (SLC26A4), which quantitative RT-PCR and immunohistochemical analysis showed to be expressed in Calu-3 cells. Fluid secretion studies showed that forskolin enhanced secretion nearly fourfold (20.0±1.0 to 73.7±3.0 μl/cm^2/24hrs, n=4), which was significantly reduced by GlyH-101 (~60%) and much lower in the CFTR knock down Calu-3 cells (~58% reduction). The forskolin stimulated secretion was more alkaline than the control secretion (7.77±0.02 viz 7.38±0.01, n=4) and had a lower Cl^- concentration (107±4mM viz 122±3, n=4), data consistent with forskolin stimulating CFTR-dependent bicarbonate secretion via a Cl^-/HCO_3^- exchange mechanism. We propose that impaired Cl^- transport, as well as defective regulation of apical anion exchangers, underlies the reduced, acidic, fluid secretion in CF airways.

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BBSRC & Novartis Institutes for Biomedical Research

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C11

Electrogenic and electroneutral HCO_3^- secretion in the possum ileum, a model HCO_3^- secreting epithelium

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In the possum (*Trichosurus vulpecula*) ileum the prostaglandin E_2 (PGE_2)-stimulated short circuit current (Isc) is dependent upon serosal Na^+ and HCO_3^- and inhibited by serosal 4,4'-diisothiocyano-stilbene-2,2'-disulfonate (DIDS) (Bartolo et al. 2009a), but insensitive to bumetanide and independent of Cl^- (Bartolo et al. 2009b). This suggests that in this tissue secretagogues stimulate electrogenic HCO_3^- secretion. Here we have used the pH stat technique to determine the serosal to mucosal flux of HCO_3^- (J^{sm}_{HCO3}) in short-circuited tissues and immunohistochemistry to identify the potential transporters involved in the secretory response. Animals were euthanased by an intracardiac injection of barbiturate administered under halothane-induced anesthesia. Ileal tissue was collected and either stripped of the underlying connective tissues and mounted in the Ussing chamber for pH stat studies or fixed for immunohistochemistry. In NaCl Ringer's, the spontaneous short-circuit current (Isc) was 3.0 ± 0.5 μEq.cm^{-2}.h^{-1} and the J^{sm}_{HCO3} was 5.6 ± 0.9 μM.cm^{-2}.h^{-1} (All values X±SEM., n=8). Replacement of all but 20 mM of the Ringer's Cl^- with gluconate had little effect on the Isc (3.0 ± 0.3 μEq.cm^{-2}.h^{-1}) but reduced J^{sm}_{HCO3} to 0.4 ± 0.1 μM.cm^{-2}.h^{-1} and total replacement of Cl^- with gluconate completely inhibited J^{sm}_{HCO3} (0.1 ± 0.1 μM.cm^{-2}.h^{-1}). In low NaCl Ringer's PGE_2 (1μM serosal) stimulated the Isc and J^{sm}_{HCO3} by 1.6 ± 0.2 μEq.cm^{-2}.h^{-1} and 1.0 ± 0.2 μM.cm^{-2}.h^{-1}, respectively, significantly greater ($P<0.05$, Student's *t* test) than the change in Isc (0.1±0.1 μEq.cm^{-2}.h^{-1}) and J^{sm}_{HCO3} (0.2±0.2 μM.cm^{-2}.h^{-1}) in unstimulated control tissues. The PGE_2-stimulated Isc was inhibited by mucosal N-(2-Naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene) glycine hydrazide (IC_{50} = 22μM) and 5 nitro-2-(3-phenylpropylamino) benzoic acid (IC_{50} = 29μM), but not 1 mM DIDS. Immunoreactivity for the cystic fibrosis transmembrane conductance regulator (CFTR) was evident in the apical membrane of cells in the crypts and lower villi of the ileal epithelium, whereas the Cl/HCO_3 exchangers, SLC26A3 and A6, where present in the apical membrane of the villous cells. These data indicate that in the possum ileum both electroneutral and electrogenic HCO_3^- secretion occur and HCO_3^- secretion accounts for the bulk of the PGE_2-stimulated Isc, which is most likely mediated by CFTR.

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C12

Direct effects of erythropoietin on iron absorption by human intestinal epithelial cells

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Enhanced erythropoiesis in iron deficiency anaemia (IDA) increases the body's demand for iron. Erythropoietin (Epo) levels are increased in IDA and are thought to act as the main homonal stimulus for red blood cell production. The presumed mode of action of Epo is via inhibition of hepatic hepcidin production which in turn increases iron absorption by intestinal enterocytes and permits iron release from reticuloendothelial macrophages. However, it is also possible that Epo signals the body's erythroid requirements directly to the enterocytes. To investigate this possibility, we have studied the effects of Epo on intestinal iron absorption in human intestinal Caco-2 cells. Caco-2 cells were grown on Transwell inserts for 17 days prior to experimentation. Epo (1U/ml) was added to the basolateral

medium and iron uptake was measured 24 h later. Parallel plates of Epo-treated cells were processed to provide membrane protein and total RNA for the measurement of iron transporter expression. Data were analysed using Student's unpaired t-test or by one-way ANOVA and Tukey's post hoc test where appropriate (significant at $p<0.05$).
Iron uptake across the apical membrane (+22%) of Caco-2 cell monolayers and efflux across the basolateral membrane (+171%) were both significantly enhanced in Epo-treated cells ($p<0.05$). These transport effects were associated with significant increases ($p<0.05$) in DMT1, ferroportin and hephaestin protein (DMT1, +53%; FPN, +64%; Heph, +58% compared with untreated control cells) and mRNA expression (DMT1, +81%; FPN, +84%; Heph, +183% compared with untreated control cells) in Caco-2 cell exposed to Epo. There was no significant change in Dcytb expression.
Our data suggest that Epo can act directly on intestinal epithelial cells to increase dietary iron absorption for subsequent utilisation in the bone marrow. These effects are mediated by Epo receptors, which are expressed on both Caco-2 cells and rat duodenal enterocytes. These data provide a novel insight into the mechanisms by which the body senses and responds to changes in the erythroid requirements for iron.

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C13

Ferroportin 1 is expressed on basolateral membranes of kidney proximal tubule cells and iron excess increases FPN1 trafficking to the membrane

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Ferroportin 1 (FPN1) is an iron exporter protein expressed in liver and duodenum, as well as in reticuloendothelial macrophages. Previously, we have shown that Divalent Metal Transporter 1 (DMT1) is expressed in late endosomes and lysosomes of the kidney proximal tubule (PT), the nephron segment responsible for the majority of solute reabsorption (1). We suggested that following receptor mediated endocytosis of transferrin filtered by the glomerulus, DMT1 exports iron liberated from transferrin into the cytosol. FPN1 is also expressed in the kidney yet its role remains obscure. As a first step towards determining the role of renal FPN1 we localized FPN1 in the rat PT. FPN1 was found to be located in association with the basolateral PT membrane and within the cytosolic compartment.FPN1 was not expressed on the apical brush-border membrane of rat PT cells. These data support a role for FPN1 in vectorial export of iron out of mammalian PT cells. Furthermore, under conditions of iron loading of cultured rat PT cells, FPN1 was trafficked to the plasma membrane suggesting a coordinated cellular response to export excess iron and limit cellular iron concentrations.

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C14

Differential methylation of the SLC30A5 zinc transporter gene in cancerous compared with normal human colon epithelium

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The SLC30A5 gene encodes the bi-directional zinc transporter ZnT5, which is expressed at the apical enterocyte membrane (Cragg et al., 2005; Valentine et al., 2007). The presence of a CpG island in the SLC30A5 promoter suggests that expression may be regulated by DNA methylation. Aberrant DNA methylation has been implicated in the aetiology of cancer. Zinc dyshomeostasis in cancer cells, including abnormal zinc transporter expression (Taylor et al., 2007), has been reported to contribute to disease progression. We hypothesise that aberrant methylation of the SLC30A5 promoter in colon tumour cells may contribute to zinc dyshomeostasis through altered ZnT5 expression.
A demethylated SLC30A5 promoter-reporter construct (Jackson et al., 2007) was generated by propagation through the methylase-deficient E. coli strain K12 ER2925 and an aliquot was methylated using SssI methylase. Caco-2 cells were transfected with these plasmids and β-galactosidase reporter gene activity was measured after 24 h. All data are stated as mean ± S.E.M in arbitrary units. The reporter gene was expressed from the unmethylated, but not the methylated, construct ($P<0.001$ by one way ANOVA followed by Tukey's multiple comparison test; 1.00 ± 0.03 for the unmethylated construct, 0.29 ± 0.05 for the methylated construct, 0.18 ± 0.03 for negative control (vector only); $n = 23$), indicating that methylation of the SLC30A5 promoter suppresses gene expression. In contrast, reporter gene expression from an equivalent construct incorporating a promoter without a CpG island (mouse Srpx2) was not affected by methylation ($P>0.05$ by Student's t-test; 1.00 ± 0.18 for the unmethylated construct, 0.87 ± 0.05 for the methylated construct; $n = 6$).
Methylation of the SLC30A5 promoter in 23 samples of human colon tumour tissue and from paired samples taken at least 10 cm away from the tumour site was measured by Pyrosequencing. Methylation was consistently lower in tumour than

in corresponding normal tissue and this effect reached statistical significance in 3 of the 21 CpG sites analysed (P<0.05 by Student's paired t-test; 69% compared with 51% (mean) for the CpG site at position –753 relative to the start of transcription, 91% compared with 62% for the site at position -606, and 75% compared with 58% for the site at position -458, n=5-23).
We have established in vitro that methylation of the human SLC30A5 promoter region results in reduced expression of an associated reporter gene and that methylation of CpG sites in the SLC30A5 promoter in colon tumour tissue was consistently lower than in adjacent normal tissue. A proposed, but unproven, resulting increase in ZnT5 expression may contribute to zinc dyshomeostasis in tumour cells and may be a factor contributing to cancer development and/or progression.
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C15

A simple model for nephrolithiasis: *Drosophila* Prestin (Slc26a5) functions as a Cl^-/Oxalate exchanger in the gut with kinetics similar to mammalian Slc26a6

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The rosy mutants of *Drosophila melanogaster* recapitulate the features of xanthinuria type I, an autosomal recessive disease of purine metabolism, with xanthine nephrolithiasis (kidney stones) of the Malpighian (renal) tubules (Wang et al., 2004; Kamleh et al., 2008). It is thus of interest to establish whether the more common calcium oxalate nephrolithiasis can also be modeled in this organism. When fed on sodium-oxalate enhanced diet, flies develop more slowly; and the tubule lumen becomes occluded with crystalline inclusions, suggesting that oxalate handling, and stone formation, may be similar in flies and humans. SLC26 transporters are anion transporters with diverse substrate specificity. They are expressed mainly in epithelia and play a central role in secretion and absorption of anions such as chloride, oxalate (ox^{2-}), sulfate (SO_4^{2-}), bicarbonate (HCO_3^-), iodide, formate and hydroxyl. Slc26a6 is expressed in kidney and gut epithelia, and mediates electrogenic Cl^-/ox^{2-}, Cl^-/HCO_3^- or Cl^-/SO_4^{2-} exchange. Slc26a6 seems to be the dominant Cl^-/ox^{2-} exchanger of the proximal tubule brush border and small intestine, and Slc26a6 knockout mice show Ca oxalate nephrolithiasis (Sindic et al., 2007; Soleimani, 2008). Recently, the chicken and zebrafish Slc26a5 transporters (prestins) were shown to mediate electrogenic Cl^-/ox^{2-} and Cl^-/SO_4^{2-} exchange rather than being an auditory sensor. The FlyAtlas.org expression resource (Chintapalli et al., 2007) reports that *Drosophila* prestin (dPrestin) is expressed mainly in the gut and tubules, rather than in sensory or nervous tissue. When expressed in Xenopus oocytes, dPrestin mediates electrogenic Cl^-/ox^{2-} and Cl^-/SO_4^{2-} exchange, similar to mouse Slc26a6. Furthermore, dPrestin can mediate electrogenic Cl^-/HCO_3^- exchange. It appears that dPrestin (like mammalian Slc26a6) is associated with gut and "renal" ox^{2-}, SO_4^{2-} and HCO_3^- transport. These results lead us to speculate that *Drosophila* may be a useful genetic model to study the factors regulating and competing with ox^{2-} transport and the formation of kidney stones.

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C16

Voltage-dependence of H^+ absorption in the larval posterior midgut epithelium of *Drosophila*

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The midgut epithelium of *Drosophila* has recently attracted attention on two fronts: the identification of regenerative cells (1-3) with a developmental plan analogous to mammalian gastrointestinal crypts, and identification in both larval and adult forms of at least three architecturally distinct segments, two of which transport acid and base (4,5). The larval posterior midgut is particularly powerful in secreting base into the lumen where the pH can exceed 10 in the steady state. This is achieved by extrusion of H^+ across the basal membrane of the epithelium into the haemolymph by an electrogenic H^+ V-ATPase, located in or very close to the basal membrane (4,5). The epithelium is tight, and the basal membrane is ≈ 8-fold more resistive than the apical, enabling voltage-clamp of this tubular epithelium to study the steady-state extrusion of H^+ as a

function of individual cell membrane potential. The epithelium was cannulated at both ends, perfused as described (4) with control Ringer solution and connected to electrometers for measurement of transepithelial parameters. The extracellular solution was scanned orthogonal to the basement membrane with a liquid ion-exchanger pH microelectrode to detect the pH profile adjacent to the basement membrane(pH_o); intracellular pH (pH_i), corrected for membrane potential, was also measured. Current was injected into the perfusion end (potential V_p) of the tubule with a bridge-balance electrometer and the small attenuation of the voltage at the collection end (V_c) was used to calculate transepithelial resistance (R_t) and short-circuit current (I_{sc}) using terminated cable analysis. Paired data±SEM are given below. In control Ringer the lumen-negative V_t was -35.6 ± 1.1mV, R_t was 1167± 85 Ω.cm^2 and I_{sc} was 34.41 ± 1.9 μA.cm^{-2} (n=34). Supplementation of the control Ringer with a 2:1 mixture of Ringer and Schneider insect medium (Sigma) prolonged viability, increased V_t to -45.4 ± 1.3mV, decreased R_t to 847 ± 43 Ω.cm^2, increased I_{sc} to 58.5 ± 3.4 μA.cm^{-2} (n=34, p<0.01) and increased the resistance ratios of the basal to apical membranes from 5.23 ± 1.0 to 21.5 ± 4.3 (n=8, p<0.01). Injection of -120, -200 and -300 nA currents to the perfusion end hyperpolarized V_c by -21 ± 8, -33 ± 6, -54 ± 5mV respectively and increased pH_o by 0.15 ± 0.01 (n=3), 0.38 ± 0.04 (n=5) and 0.14 ± 0.03 (n=5) units respectively. Injection of 180 ± 40nA (n=3) hyperpolarized V_c by -34 ± 6mV and increased pH_i by 0.34 ± 0.14 units (n=3). We conclude that the hyperpolarization of V_t reduces the H^+ efflux by the H^+ V-ATPase (increase of pH_o) and also increases backflux into the lumen through non-selective channels in the apical membrane (as evidenced by increase in pH_i).

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C17

Epidermal Growth Factor chronically regulates chloride secretion across colonic epithelial cells

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Cl^- secretion, the primary driving force for intestinal fluid secretion, is dysregulated in many intestinal disorders, leading to the clinical manisfestation of diarrhoea. Growth factors, such as epidermal growth factor (EGF), are important regulators of epithelial Cl^- secretion and previous work from our laboratory has shown that EGF chronically enhances colonic epithelial secretory function.
Aim: To investigate molecular mechanisms underlying EGF potentiation of colonic epithelial secretory capacity. Monolayers of T_{84} cells, were mounted in Ussing chambers and Cl^- secretion measured as changes in short-circuit current (I_{sc}). Transport protein expression was measured by RT-PCR. Results are expressed as mean±sem for a series of n experiments. Statistical analyses were made by paired Student t-test or one way ANOVA amd Newman-Keuls post-test. p values<0.05 were considered to be significant. Results: Acute treatment with EGF (100 ng/ml for 15 min) induced increases in mRNA expression of key components of the Cl^- secretory pathway, including CFTR (270±20% of control; n=3; p<0.01), NKCC1 (338±90% of control; n=3; p<0.001), KCNN4 (150±20% of control; n=5; p<0.05) and the Na/K-ATPase β_1 subunit (370±100% of control; n=4; p<0.01). EGF also increased mRNA levels of transmembrane protein 16A (TMEM16A) (256±45% of control; n=6; p<0.01) a recently identified protein associated with Ca^{2+}-dependent Cl^- channel activity. To determine signaling pathways involved in mediating the effects of EGF, we examined the effects of PKC (GF 109203X), PI3-K (LY294002), ERK (PD98059) and p38 MAPK (SB203580) inhibitors on the ability of the growth factor to enhance I_{sc} responses to either the Ca^{2+} or cAMP-dependent secretagogues, carbachol(CCh) and forskolin(FSK) (Table 1). EGF chronically increases colonic epithelial secretory function through upregulation of key transport proteins that comprise the Cl^- secretory pathway, including TMEM16A, a novel Ca^{2+}-dependent Cl^- conductance that has not previously been shown to be expressed in colonic epithelia. Signaling pathways underlying EGF regulation of transport protein expression are complex and involve multiple effector proteins such as MAPKs, PKC, and PI3-kinase. Our data suggest that modulation of EGFR-dependent signaling pathways may prove a useful approach in therapy of intestinal disorders associated with dysregulated epithelial transport.

Inhibitor	CCh (100μM)		FSK (10μM)	
	+ EGF	+ EGF + inhibitor	+ EGF	+ EGF + inhibitor
GF 109203X (5μM)	206±31**	107±13	148±10**	115±11
LY290042 (25μM)	188±30**	134±14	147±8**	117±14
PD98059 (25μM)	155±8***	120±15	153±17**	107±6
SB203580 (10μM)	188±30***	162±21**	147±8**	110±13

Table1. I_{sc} responses to CCh and FSK were measured 24 hrs after pretreatment with EGF. Data are expressed as % control response to CCh (n=6) or FSK (n=5) alone (**p<0.01; ***p<0.001).

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C18

Cystic fibrosis transmembrane conductance regulator-dependent expression of SLC26A7 in the thyroid

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The *SLC26A4* gene encodes Pendrin, an anion transporter believed to underlie I^- exit into the thyroid follicular lumen, a critical initial step in thyroid hormone production. Pendrin mutations are linked to Pendred syndrome, a human disease characterized by deafness and, occasionally, hypothyroid goiter. However, the *Slc26a4*$^{-/-}$ mouse is systemically euthyroid, suggesting that other anion transport proteins contribute to I^- efflux [1].

One candidate is Slc26a7, an anion channel capable of conducting I^- that is found in the kidney and the stomach [2,3]. Gene microarray analysis showed strong presence of two *SLC26A7*-specific probes in mouse thyroid (n = 3 replicates). Subsequent mining of mouse tissue GEO database records indicated that thyroid *SLC26A7* levels exceed those present in kidney and stomach. The rabbit polyclonal anti-Slc26a7 antibody, 968,[4] recognised two bands, one migrating at ~ 85 kDa and another at ~ 65 kDa in samples of mouse total thyroid lysate (n = 3); bands at the identical position were detected in Slc26a7-injected, but not water-injected, *Xenopus* oocyte lysates.

Because subclinical hypothyroidism has been documented for cystic fibrosis (CF) patients, we tested whether the cystic fibrosis transmembrane conductance regulator (CFTR) mediates thyroid I^- transport *via* effects on SLC26A7. We established a novel porcine thyroid model for CF. Thyroid glands from $CFTR^{+/+}$, $^{+/-}$ and $^{-/-}$(wt, het, ko) piglets were shipped overnight on ice from the laboratory of Prof. M. Welsh, U.Iowa, HHMI [5]. Glands were divided for 1) primary culture, 2) preparation of total tissue protein lysates or 3) fixation with 4% paraformaldelhyde for preparation of paraffin sections. Gross histology and transepithelial resistance did not differ significantly between wt, het and ko thyroids (wt: 2394 ± 753 $\Omega \cdot cm^2$; het: 2617 ± 1147 $\Omega \cdot cm^2$; ko: 3842 ± 1005 $\Omega \cdot cm^2$; mean ± sem; n = 3 monolayers; $p > 0.05$ between all groups; paired t-tests). A mouse monoclonal anti-Slc26a7 (14H5; Abcam) recognised the 85 kDa, but not the ~65 kDa, band in $CFTR^{+/+}$ and $^{+/-}$ pig thyroid lysates. Conversely, rabbit polyclonal antibody 968 showed the 65 kDa band, but did not detect the 85 kDa band. CFTR knockout markedly decreased the 85 kDa species (n = 3). These findings suggest a functional relationship between CFTR and SLC26A7. Future work will include measurement of I^- secretion by primary thyroid cultures derived from $CFTR^{+/+}$, $^{+/-}$ and $^{-/-}$ pigs, as well as by $CFTR^{-/-}$ thyroid monolayers with knock-in of *SLC26A7*, or $CFTR^{+/+}$ with knock-down of *SLC26A7*. Possible functional coupling of CFTR and SLC26A7 *via* PDZ-domain interactions also will be explored.

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C19

Spatial segregation of calcium release and uptake in secretory epithelium

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Store operated calcium entry (SOCE) is a ubiquitous mechanism that ensures the refilling of the calcium stores, mainly the endoplasmic reticulum (ER) after depletion (1). A reduction of ER calcium content is sensed by STIM1 protein which translocates into subplasmalemmal puncta upon store depletion where it activates Orai channels and triggers calcium influx (2). In the pancreatic acinar cell, a well established model of exocrine secretory epithelium, enzyme secretion is driven by localised calcium oscillations. The main Ca^{2+} release sites – IP_3 receptors - are located in the apical region of the ER (3).

Our experiments on primary murine pancreatic acinar cells show that when the ER calcium store is depleted by continuous physiological or pathological stimulation STIM1 translocates towards the basal and lateral membranes only avoiding the apical pole. This indicates that different ER compartments are specialised in separate Ca^{2+} handling functions. Spatial segregation of intracellular Ca^{2+} release and Ca^{2+} reloading ensures minimal interference and maximum efficiency of both processes in this highly specialised cell type.

The most controversial aspect of this finding is that in pancreatic acinar cells the basal and lateral regions contain rough endoplasmic reticulum. The distance permissive for physical interaction between STIM and Orai proteins is significantly smaller than the size of an average ribosome that decorates the surface of the ER in these cells (4). We resolved this paradox by identifying ribosome free ER terminals forming junctions between the plasma membrane and the rough ER in the basolateral regions.

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

C20

Non-conjugated bile acids induce mitochondrial damage and inhibit bicarbonate transport mechanisms in pancreatic duct cells

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Introduction. We have recently shown that a high dose (1mM) of the non-conjugated bile acid chenodeoxycholate (CDC) had strong inhibitory effects on the activities of acid/base transporters on pancreatic ductal epithelial cells (PDEC) (Ref 1). However, the same dose of conjugated glycochenodeoxycholate (GCDC) had no effect on the ion transporters. In addition, a high dose of CDC evoked a toxic sustained calcium elevation. Our aim was to characterize the intracellular mechanisms by which the ion transport mechanisms are inhibited.

Methods. Intra/interlobular pancreatic ducts were isolated from the pancreas of guinea pigs. High dose (1mM) of CDC or GCDC was administered basolaterally for 1-10 minutes. Intracellular pH (pH_i) of PDEC were measured by microfluorometry using BCECF. The intracellular ATP level $(ATP)_i$ was determined using Mg-Green which has been shown to indirectly reflect the changes in $(ATP)_i$ (Ref 2). Morphological changes of PDEC were evaluated by transmission electron microscopy.
Results. Administration of a low dose (0.1mM) of CDC or GCDC for 10 min had no effects on the intracellular organelles. In addition, a high dose (1mM) of the conjugated GCDC did not induce morphological changes. Importantly, exposure of 1mM CDC for 10 min strongly damaged all of the mitochondria. The mitochondria swelled up and their inner membranes were disrupted. Other intracellular organelles such as nuclei or Golgi apparatus seemed to be unaltered. For positive control experiments we used the mitochondrial toxin carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 100μM) which totally mimicked the effect of CDC on mitochondria. Next we set out to investigate whether $(ATP)_i$ is affected due to the mitochondrial damage. Administration of a low dose of CDC or GCDC for 10 min had no effect on the $(ATP)_i$, however, a high dose of CDC and CCCP markedly and irreversibly reduced the $(ATP)_i$. Although 1mM GCDC also decreased $(ATP)_i$, this depletion was reversible and significantly less than the depletion caused by CDC or CCCP. Finally, we provided evidence that $(ATP)_i$ depletion is crucial in the toxic inhibitory effect of CDC on the ion transporters. To characterize the effects of $(ATP)_i$ depletion on the activities of NHE, NBC and CBE, we used the NH_4Cl pulse technique in HCO_3^--buffered solution. CCCP strongly inhibited NBC, NHE (recovery from acid load) and CBE (recovery from alkali load).
Conclusions. In conclusion, these results clearly show that (i) a high dose of the non-conjugated CDC, but not the conjugated GCDC, causes mitochondrial damage followed by $(ATP)_i$ depletion and (ii) the $(ATP)_i$ depletion by itself can be responsible for the impaired fluid and HCO_3^- secretion.

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

C21

Distribution of vascular connexin proteins in the airway epithelium of the rat: evidence for myo-epithelial gap junctions

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Introduction
Interendothelial and myoendothelial gap junctions are well described in the vascular system. However, little is known about the role of inter-epithelial gap junctions of the lung. The airway epithelium acts as a functional syncitium. Failure of this system is a crucial part of the pathology of diseases such as cystic fibrosis and acute respiratory distress syndrome. Furthermore, the function of the lung depends on the interaction between airway and blood vessels for gas exchange, and the pulmonary vessels and bronchi may have a common embryological origin[1]. It is hypothesised that these tissues have connexin proteins in common. This study looked at the distribution of vascular connexin proteins in the airways.
Methods
Slices from the first intrapulmonary bronchus and peripheral bronchioles (50-100μm diameter) of the rat were dual-labelled using connexin (Cx) 37, 40 or 43 antibody and α-actin antibody, and visualized for confocal microscopy using AF488 and AF594.
Results
Connexin 43 was abundantly distributed along the inter-epithelial borders of the first intrapulmonary bronchus. Connexin 37 seems absent from this large airway. Connexin 40 showed faint staining along the epithelial borders, but showed much more abundant staining along the areas of contact between the base of the epithelium and the underlying airway smooth muscle. Co-staining with α-actin demonstrated that connexin 40 in the primary bronchus was clearly associated with smooth muscle. However, connexin 40 staining was found primarily along the smooth muscle-epithelial cell border with little signal coming from deeper smooth muscle layers.
In the peripheral bronchiole, connexin 37 was found along the border between the smooth muscle and epithelium, but seemed absent in either the deeper smooth muscle border or along the borders between epithelial cells. Both connexin 40 and connexin 43 were abundantly present in the epithelial tissue of the peripheral airway.
Conclusion
To our knowledge these data represent the first demonstration of blood vessel-related connexin proteins in the respiratory epithelium of conducting airways. Although connexin 43 expression between alveolar cells has been described [2], there are almost no studies of connexin communication in the respiratory epithelial syncitium. Our data show variation in connexin proteins along the respiratory tree, with connexin 37 being absent in large airways but abundant in peripheral bronchioles. In addition there is exciting new evidence to suggest myo-epithelial gap junctions, which could modulate bronchial smooth muscle tone. The ability of the respiratory epithelium to influence bronchial smooth muscle tone was described in the past [3], but the mechanism remains unclear. Myo-epithelial junctions may form part of this mechanism.

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C22

The anti-secretory response to oestrogen in the distal colon is dependent on synergistic transcriptional and signal transduction events

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The secretion of Cl- by the epithelial cells of the distal colonic crypt provides the driving force for the movement of fluid into the luminal space of the colon. Experimental procedures were performed on colonic epithelium harvested from Sprague-Dawley rats following humane sacrifice by cervical dislocation. The distal colon was removed post mortem and cut into sections. The epithelium was then dissected away from the underlying muscle layer or else crypts were detached from the tissue by Ca2+ depletion and then isolated. 17β-estradiol (E2) stimulates a rapid and sustained suppression of this secretory process in the female but not in male rat colon. The modulation of KCNQ1 channel activity through E2-induced PKCδ isozyme and PKA isoform I activation regulates the electrochemical gradient that facilitates apical Cl- secretion. The sexual dimorphism in the E2 response is associated with elevated expression of PKCδ in female compared to the male colon tissue. The anti-secretory response to E2 is regulated throughout the rat female reproductive (oestrous) cycle and is primed by genomic regulation of the kinases. E2 (1-10 nM) decreased cAMP-dependent secretion in colonic epithelia during the oestrus, meta-oestrus and dioestrus stages of the cycle. Weaker inhibition of secretion was demonstrated in the pro-oestrus stage. The expression levels of PKCδ and PKA fluctuated throughout the oestrous cycle and correlated with the potency of E2 in suppressing secretion. E2 stimulated the up-regulation of PKCδ and PKA expression through increased transcription via a PKCδ-MAPK-CREB regulated pathway indicating a genomic priming of the anti-secretory response. PKCδ was activated by the membrane impermeant E2-BSA and this response was inhibited by the oestrogen receptor (ER) antagonist ICI 182,780. The 66kDa ERα isoform was present at the plasma membrane of female colonic crypt cells with a lower abundance found in male colonic crypts. This study demonstrates acute and chronic E2 regulation of intestinal secretion through interdependent transcriptional and signalling responses.

O'Mahony F et al. (2007). J Biol. Chem. 282, 24563-24573

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C23

Lack of restoration *in vivo* by K^+ channel modulators of jejunal fluid absorption after heat-stable *Escherichia coli* enterotoxin (STa) challenge

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The effect of glibenclamide, a chloride and potassium channel blocker, clotrimazole, a potassium channel blocker and cromokalim, a potassium channel opener were tested in vivo for their ability to prevent E.coli STa enterotoxin from inhibiting jejunal fluid absorption in the anaesthetised rat. Potassium channel opening is thought to play a central role in the maintenance of the enterocyte membrane potential that provides the correctly aligned electrochemical force needed to promote chloride ion secretion towards the intestinal lumen. Sections of ileum from rabbits post mortem were mounted in a Burn-Dale apparatus and longitudinal muscle tension was measured. The ability of these compounds to restore STa inhibited fluid absorption was examined. A perfused intestinal loop preparation was used to measured luminal uptake of fluid in vivo by volume recovery from the jejunum of the anaesthetised rat (70 mg/kg i.p. Sagatal). All procedures were carried out in conformity with UK legislation. Data are given as the mean plus the standard error of the mean with the number of loop experiments in parenthesis. Significance was tested by 't'-test.
The unchallenged jejunum absorbed fluid at a rate of 98.8 ± 6 (8) ul/cm/hr. In the STa (80 ng/ml) challenged loop, there was a significantly lesser ($p<0.001$) rate of 33.2. ± 8.1 (9) ul/cm/hr absorption, that was also significantly higher ($p< 0.001$) than zero absorption. Intraluminal 17 uM glibenclamide plus E.coli STa gave a rate of fluid absorption of 24.8 ± 8.9 (6) ul/cm/hr. Clotrimazole at 10 uM and STa gave a rate of fluid absorption of 17.0 ± 8.4 (5) ul/cm/hr. Intraluminal cromokalim of 1 uM plus STa gave a rate of 43.9 ± 11.9 (6) ul/cm/hr., not significantly different from values for STa challenge in the absence of cromakalim. Clotrimazole, glibenclamide and cromakalim did have significant effects on the cardiovascular system in vivo when given as an i.v bolus dose and also on rabbit ileum motility in vitro. Intravenous glibenclamide (250ug per 15 minutes) and clotrimazole (300 ug per 15 minutes) also failed to restore STa challenged fluid absorption. Cromakalim (10 ug per 15 minutes) worsened ($p<0.05$) STa inhibited fluid absorption to 10.3 ± 6.8 (5) ul/cm/hr. These compounds were identified as anti-secretory agents in vitro on the basis of their ability to reduce short-circuit current elevation after STa exposure back to pre-challenge levels. When tested in vivo, these compounds are unable to prevent the reduction in fluid absorption after STa challenge. These findings contradict the concept of electrogenic chloride ion secretion as the basis for diarrhoeal disease, since the pharmacology of inhibition of elevated short circuit current is not matched by an ability to restore STa challenged fluid absorption.

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C24

Mitochondria are crucial for maintaining of store-operated Ca^{2+} influx and ER refilling under prolonged acinar cell stimulation

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Store-operated Ca2+ entry is mediated by plasma membrane store-operated Ca2+ channels (SOCCs) activated due to the depletion of endoplasmic reticulum (ER) Ca2+ stores. Activation of SOCCs is required for Ca2+signaling and ER refilling which both important for the protein synthesis and exocytosis.

We measured free Ca2+ concentration in the cytosol ([Ca2+]cyt), inside the ER ([Ca2+]ER) and in mitochondria ([Ca2+]mit) using Fura-2/AM, Mag-fura-2/AM and Rhod-2/AM fluorescent dyes correspondingly.
Experiments were performed on male Wistar rats (6–7 weeks old, 100–150 g). Gland was removed after intraperitoneal injection of pentobarbital. Submandibular salivary gland acinar cells were isolated by collagenase treatment.
In the present study we first stimulated the acinar cells with acetylcholine (ACh) in Ca2+ free media (to deplete the ER and activate the store-operated Ca2+ channels (SOCCs)) and then readded Ca2+ to the media (to vizualize Ca2+entry). We showed that the amplitude and the initial rate of the ACh induced SOCC-mediated [Ca2+]i transients clearly depend on the potency and the duration of agonist stimulation. In particular, the initial slope and amplitude of [Ca2+]i transients were significantly decreased upon inhibition of mitochondria (Mit) Ca2+ uptake or Ca2+ release from the Mit. The latter indicate the decrease number of active SOCCs. In the whole cell patch-clamp experiments we directly showed that [Ca2+]mit transients are generated immediately after the SOCCs activation and their kinetics directly correlate with the potency of agonist stimulation. Notably, we found that under conditions of sustained ACh stimulation adding of BAPTA to the pipette solution caused dramatic decrease in the amplitude and the initial slope of the SOCE-induced [Ca2+]mit transients. In addition we showed that in the conditions of continious ACh stimulation the inhibition of Mit Ca2+ buffering caused almost complete inhibition of SOCCs Ca2+ influx the ER reffiling whereas upon short ACh action inhibition was much smaller.
In summary, Mit are crucial for maintaining of SOC-mediated Ca2+ entry; ii) subplasmalemmal Mit effectively grade Ca2+ entry due to prevention of Ca2+-dependent inactivation of SOCCs; iii) in the condition of sustained ACh stimulation SOCE is dramatically increased in a direct proportion to potency of ER depletion; cell adapts its Ca2+ clearance system directing more Ca2+ into mitochondria via microdomains of high [Ca2+]cyt, providing strong positive feedback on SOCE. Furthermore, in the continuous presence of an agonist, Ca2+ transport inside mitochondria and its release through the Na+/Ca2+ exchanger supply ~90% of Ca2+ for ER refilling; whereas in the absence of an agonist ~30%. Robustly increased role of mitochondria Ca2+ dynamics in the maintenance of sustained SOCE and ER refilling represents a novel function of mitochondria as an intrinsic instrument enabling long lasting secretion.

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C25

Modulation of Ca^{2+} signalling in mouse lacrimal acinar cells by Nitric Oxide

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Lacrimal and submandibular gland fluid secretion is regulated by changes in $[Ca^{2+}]_i$. We have shown previously, in both mouse and human submandibular acinar cells, that NO has a biphasic effect on Ca^{2+} signalling (Caulfield et al., 2009). The first phase of the response is a cyclic-GMP mediated amplification of the CCh-evoked Ca^{2+} signal, most likely via cyclic-ADP ribose production and activation of ryanodine receptors. The second, chronic, phase of the response to NO is a slow inhibition of the CCh-evoked signal which was not blocked by ODQ and is therefore unlikely to be mediated by cGMP. We have speculated that modulation of the Ca^{2+} signal by NO could have a role in the pathophysiology of Sjögren's syndrome, a disease of salivary hypofunction. Sjögren's syndrome affects both salivary and lacrimal glands and therefore we have hypothesized that, if NO is important in the pathophysiology of this condition, it should also modulate Ca^{2+} signaling in lacrimal gland acinar cells.
We have tested our hypothesis using isolated acinar cells, obtained by collagenase digestion of mouse lacrimal glands and maintained for 24-48 hours in primary culture. Changes in $[Ca^{2+}]_i$ were measured using Fura-2 microfluorimetry. We have measured the effects of exposure of the lacrimal acinar cells to the NO releasing agent SNAP on the magnitude of the Ca^{2+} signal elicited by repetitive CCh stimulation.
Like submandibular cells, lacrimal cells showed a pattern of first amplification and then inhibition of the Ca^{2+} signal in the presence of NO. Unlike submandibular cells, lacrimal acinar cells also showed a similar pattern of response to repetitive CCh stimulation in the absence of NO. These data show a "use dependence" to repetitive stimulation by CCh in lacrimal cells that mirrors the effect of NO seen previously in submandibular cells. One possible mechanism for the "use dependence" is endogenous production of NO by lacrimal acinar cells *in vitro*. Preliminary experiments using L-NAME to block NO production do not support this mechanism.

Caulfield VL, Balmer C, Dawson LJ & Smith PM. (2009). A role for nitric oxide-mediated glandular hypofunction in a non-apoptotic model for Sjogren's syndrome. Rheumatology (Oxford) 48, 727-733.

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

C26

Primary human bronchial epithelial cells respond to a bile acid challenge by up regulating interleukin-8 production

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Introduction: The pathophysiology of both cystic fibrosis (CF) lung disease and lung allograft injury following transplantation involves neutrophilic inflammation, which is associated with increased levels of the neutrophil chemokine interleukin(IL)-8.[1,2] Bile acid aspiration, as a result of duodeno-oesophageal

reflux, has been implicated as a common and emerging aspect of lung injury.[3] In this study we have evaluated the effect of bile acid challenge on primary bronchial epithelial cells derived from patients with CF and from lung allografts.

Methods: Cultures of primary bronchial epithelial cells were established from bronchial brushings from lung transplant recipients and from cystic fibrosis lungs removed at the time of transplantation, as described previously (see Forrest *et al.*[4] and Brodlie *et al.*[5]). Epithelial cell cultures were stimulated with different primary and secondary bile acids, namely cholic and chenodeoxycholic acid, deoxycholic and lithocholic acid and taurodeoxycholic and taurocholic acid, at the physiologically achievable concentrations of 1, 5 10, 25 and 50 μmol/L. IL-8 was then measured in the culture supernatants by enzyme-linked immunosorbent assay at 48 hours post-stimulation (MesoScale Discovery). Cell viability was assessed at the end of each experiment (CellTiter-Blue, Promega). Differences in IL-8 production from control, unstimulated conditions were compared using a non-parametric paired t-test (Prism, GraphPad).

Results: Detailed results are presented for stimulation with lithocholic acid as this was the most effective. Stimulation of bronchial epithelial cells from people with CF (n=7) and lung allografts (n=7) with lithocholic acid at 1, 5, 10 and 25 μmol/L resulted in a significant increase in IL-8 production compared to control, unstimulated conditions ($p<0.05$). Treatment with 50 μmol/L lithocholic acid resulted in >90% cell death. Figure 1 displays IL-8 production, expressed as increase from unstimulated conditions, by bronchial epithelial cells from people with CF and lung allografts following treatment with different concentrations of lithocholic acid.

Conclusion: Primary bronchial epithelial cell data may be used to explore potential mechanisms in the pathogenesis of CF lung disease and lung allograft injury. Airway epithelial cells up regulate IL-8 production when exposed to lithocholic acid and our data indicate a possible mechanism whereby aspiration of bile acids, as a result of duodeno-oseophogaeal reflux, may be linked to neutrophilic inflammation in CF lung disease and lung transplant injury. The translational significance of this finding is that reflux may be treated and essentially prevented by fundoplication procedures.

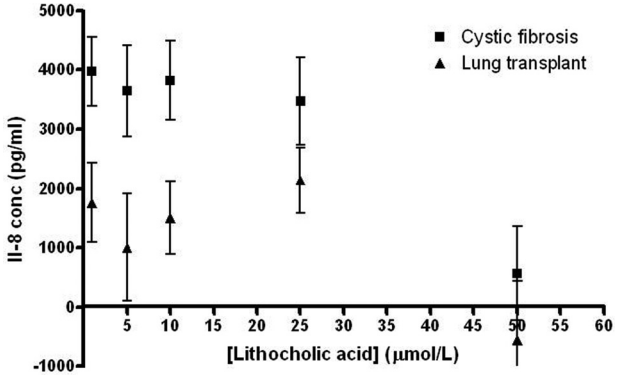

Figure 1. Increase in IL-8 production from baseline

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Brodlie M *et al.* (2009) *Experimental Lung Research* In Press

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

PC1

Store-operated calcium entry into the secretory cells of *Drosophila melanogaster* larval salivary glands

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The major Ca^{2+} entry pathway in electrically nonexcitable cells is store-operated, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx [1, 2]. We have identified the *olf186-F* gene which encodes the Orai protein as a component of the store-operated Ca^{2+}-channel in the plasma membrane of the secretory cells of *Drosophila melanogaster* larval salivary glands[3].

The main aim of these investigations was to study the mechanisms underlying the activation of store-operated Ca^{2+}-entry in these organs. For this purpose, we used Drosophila strain that expresses genetically encoded Ca^{2+}-sensor G-CaMP1.6 in larval salivary glands. Salivary glands were dissected under the HEPES-buffered physiological saline solution. The passive depletion of intracellular Ca^{2+}-stores was promoted by applying of 10 μM ionomycin and/or 10μM thapsigargin. Ca^{2+}-related variations of G-CaMP1.6 fluorescence were studied using a confocal microscopy.

To determine whether thapsigargin mobilizes Ca^{2+} from intracellular stores, secretory cells were perfused with EGTA-containing medium and then exposed to 10 μM thapsigargin. Resting G-CaMP1.6 fluorescence intensity in single secretory cells was 33.21 ± 2.33 conventional units (n = 51) in 0-Ca^{2+} medium. The addition of thapsigargin caused a non-transient increase in the G-CaMP1.6 fluorescence intensity in all cells observed (n = 51, mean = 44.44 ± 3.64 c.u., $P \leq 0.05$). The re-admission of external 2 mM Ca^{2+} resulted in a significant elevation of the G-CaMP1.6 fluorescence intensity (n = 51, mean = 85.97 ± 10.29 c.u., $P \leq 0.001$). Additionally, there was some variability among cells in the rate of rise of the G-CaMP1.6 fluorescence intensity and the magnitude of the peak achieved. Ca^{2+} ionophores, e.g. ionomycin, can also be used to promote the passive depletion of intracellular Ca^{2+} stores, although they also directly transport Ca^{2+} across the plasma membrane [4]. Drosophila salivary glands treatment by ionomycin (10 μM) in Ca^{2+}-free medium caused a non-transient increase in the G-CaMP1.6 fluorescence intensity by 11 % (n = 30, $P \leq 0.01$). After the re-admission of 2 mM Ca^{2+} all secretory cells (n = 30) exhibited synchronous Ca^{2+} transients and the G-CaMP1.6 fluorescence intensity elevated by 89 % (n = 30, $P \leq 0.001$). This could result from Ca^{2+} entering into the cells under the Ca^{2+}/H^{+}-exchange and/or the store-operated Ca^{2+}-entry activation upon the emptying of the intracellular store.

Therefore, thapsigargin mobilizes Ca^{2+} from intracellular storage sites and inhibits its uptake by the endoplasmic reticulum Ca^{2+} stores. The activation of the store-operated Ca^{2+} entry across the plasma membrane after depletion of Ca^{2+} stores could suggest about its role in the maintenance of Ca^{2+} homeostasis in the secretory cells of *Drosophila melanogaster* larval salivary glands.

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PC2

Effect of TNFa on a-methyl-glucoside, glutamine and phenylalanine uptake by monolayer of Caco-2 cells

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TNFα levels are increased during intestinal inflammation processes and malabsorption of nutrients may occur. We have previously demonstrated that TNFα inhibits galactose absorption in rat and rabbit intestinal everted rings and other authors have shown regulation of PET1, TAUT and SERT transporters by the cytokine in Caco-2 cells. Thus, the aim of the present work was to investigate in Caco-2 cells the effect of TNFα on the absorption of alpha-methyl-D-glucoside (MG), glutamine and phenyalanine, and the possible implication of PKA and PKC.

Caco-2 cells were grown on plates (access of the cytokine from the apical membrane) or on filters (access from both apical and basal membranes). Cells were preincubated for diverse times with different concentrations of TNFα in the basal or apical medium, before measuring the uptake of the radiolabeled substrate during 15 min. In the experiments with kinases inhibitors, cells grown on plates were preincubated for 30 min with 2 μM chelerythrine (PKC inhibitor) or 1 μM H-89 (PKA inhibitor) prior to the 15 min incubation with 25 or 50 ng/ml TNFα. MG was chosen as specific substrate of the Na^{+}/glucose cotransporter SGLT1, Phe as specific substrate of $B^{0}AT1$ and Gln as substrate of both ASCT2 and $B^{0}AT1$.

The results with plates-grown cells demonstrated inhibitory effect of TNFα (with 10 ng/ml and higher concentrations) on 0.1 mM MG uptake, even without preexposure to the cytokine. After 24 h preincubation, the inhibition was observed already with 1ng/ml. When filters were used, the presence of 10 or 25 ng/ml TNFα in the basal medium during 1h preexposition increased MG uptake, whereas after 24 h, 1 ng/ml inhibited uptake and no effect was found with 10 ng/ml TNFα. The apical presence of TNFα (10, 25 or 50 ng/ml) diminished the uptake of 0.1 mM Gln and 0.1 mM Phe by Caco-2 cells grown on plates. In filter-grown cells, the presence of TNFα (10-50 ng/ml) in the basal compartment during 1, 6 or 24 h preincubation did not modify the uptake of Gln or Phe.

In studies performed using plates, both H-89 and chelerythrine reversed the inhibition of MG uptake due to 25 ng/ml TNFα whereas they increased the inhibition of Gln and Phe uptake due to the cytokine.

In summary, TNFα inhibits SGLT1, B^0AT1 and probably ASCT2 function in monolayer of Caco-2 cells. The effect is stronger when the cytokine is acting from the apical membrane. Both PKA and PKC seem to be involved in the apical effect of the cytokine on SGLT1.

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

PC3

Luminal leptin regulates the activity of sugar and amino acid transporters in Caco-2 cells

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We have previously demonstrated that leptin inhibits the sugars absorption in rat intestine *in vitro* and *in vivo* acting from the lumen, with the involvement of PKA and mainly PKC. We also found that leptin receptors are expressed in both the apical and basolateral membrane of enterocytes. In the present work we have studied the effect of leptin on the absorption of α-methyl-glucoside (MG), glutamine and β-alanine by the human intestinal epithelial cell line Caco-2 and the possible implication of PKC and PKA.

The Caco-2 monolayer was formed on plates (access from the apical membrane) and filters (access from the basolateral side). The apical uptake of the substrate was measured in the absence or presence of leptin (0.2 and 8 nM) after 5, 15 and 30 min incubation. The involvement of PKA or PKC was studied by preincubating the cells for 30 min with the respective inhibitors H-89 (1 μM) or chelerythrine (chel) (2 μM).

Leptin present in the apical side inhibited by 25 % the uptake of 0.1 mM MG (substrate of SGLT1) after 5 and 30 min. Interestingly, after 15 min, 0.2 nM leptin did not modify sugar uptake whereas 8 nM increased it by 20 %. Both H-89 and chel reversed this increase, indicating the implication of PKA and PKC on leptin effect. At 5 and 30 min, however, the inhibitors seemed to potentiate the decreasing effect of the hormone. On the other hand, leptin present in the basal compartment inhibited, by 30 %, MG uptake only at 8 nM after 30 min.

Absorption of 0.1 mM β-Ala was measured at pH 6 in the presence of Na^+, which is the optimal condition for the activity of its transporter PAT1. The effect of apical leptin on β-Ala uptake followed the same pattern as for MG at the three incubation times. However, leptin acting from the basolateral side did not modify the amino acid uptake. Leptin inhibition of β-Ala uptake at 30 min was completely reversed by H-89, indicating the involvement of PKA, whereas chel did not show any effect.

Finally, uptake of 0.1 mM Gln (substrate of ASCT2 and B^0AT1) was also inhibited 40% by apical leptin at 5, 15 and 30 min and by basolateral leptin after 15 min (15%). As it happened for MG, both H-89 and chel increased leptin inhibitory effect at 30 min, indicating that neither PKA nor PKC are implicated in leptin action at this incubation time.

In summary, leptin regulates the activity of sugar and amino acid transporters in Caco-2 cells, acting mainly from the apical membrane, with implication of PKA and PKC.

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PC4

A role for nucleoside diphosphate kinase B (NDPK-B) in the regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelia

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In airway epithelia, the phosphorylation of nucleoside diphosphate kinase (NDPK) is regulated by [chloride (Cl^-)] and [Na^+]. NDPK-B is a histidine kinase and has been shown to interact with the cystic fibrosis transmembrane conductance regulator (CFTR) in a cAMP-dependent manner. The aim of this study was to investigate whether the interaction between NDPK-B and CFTR was relevant to the function of CFTR. Cell stimulation with 3-isobutyl-1-methylxanthine (IBMX)/forskolin resulted in the translocation of NDPK-B from the cytosol to the membrane, and Western blot analysis of CFTR immunoprecipitates showed association of NDPK-B and CFTR, which was significantly increased with IBMX/forskolin stimulation. Over-expression of GFP-tagged WT NDPK-B proteins in the human bronchial epithelial cell line 16HBE14o- resulted in a significant increase in the CFTR-mediated whole cell conductance; the outward

conductance increased from 241 +/- 148 μS/cm^2 to 1844 +/- 533 μS/cm^2 (P < 0.05, unpaired t-test), and inward conductance increased from 259 +/- 193 μS/cm^2 to 1542 +/- 537 μS/cm^2 (P< 0.05, unpaired t-test). These interactions are attenuated by a NDPK-B peptide corresponding to amino acids 36 – 54 of the protein. These data suggest that NDPK-B may be important for the signaling processes that regulate CFTR function in epithelial cells.

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Dr. Louise Robson and Dr. Richmond Muimo, University of Sheffield

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PC5

The effects of a nominally selective PKB inhibitor (Akt-I1/2) on insulin-induced Na^+ absorption in mpkCCD renal epithelial cells

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Na^+ re-absorption within the distal nephron determines the amount of Na^+ lost in the urine and this process can be controlled by insulin, a hormone that activates phosphoinositide-3-kinase (PI3K) (Loffing and Korbmacher, 2009). The mechanism permitting this control is not well understood but data from several sources highlight the importance of serum and glucocorticoid-inducible kinase 1 (SGK1), a kinase activated by PI3K that can control the apical abundance of epithelial Na^+ channels (ENaC) (Loffing and Korbmacher, 2009). However, studies of fisher rat thyroid cells expressing the ENaC subunits suggest that insulin-induced Na^+ absorption depends upon protein kinase B (PKB, also known as Akt), another target of PI3K (Lee *et al.*, 2007). The present study therefore explores the effects of Akt-I1/2, a compound reported to inhibit PKB selectively, upon insulin-induced Na^+ transport in a cell line (mpkCCD) derived from the distal nephron (Bens *et al.*, 1999). Transepithelial Na^+ transport was studied using standard Ussing chamber techniques whilst changes in PKB activity were assessed using Western analysis to monitor the phosphorylation of PKB-Ser^{473}, a residue critical to the PI3K-dependent activation of this kinase. Cellular SGK1 activity was monitored by assaying the phosphorylation of residues within an endogenous protein (n-myc downstream gene regulated protein 1, NDRG1-Thr$^{346/356/366}$) that are phosphorylated by SGK1 but not by other kinases (Murray *et al.*, 2005). Data are mean ± s.e.m and values of n refer to the number of times a protocol was repeated using cells at different passage.

Akt-I1/2 (1 μM, 60 min) caused ~25% inhibition of the amiloride-sensitive I_{SC} measured under basal conditions(Akt-I1/2: -15.8 ± 1.4 μA^{-2}, solvent vehicle: -20.4 ± 1.0 μA^{-2}, n = 15, P < 0.02, Student's paired t test). Although insulin (20 nM, 60 min) consistently augmented this current, the response measured in Akt-I1/2-treated cells (ΔI_{SC}: -4.9 ± 1.9 μA cm^{-2}, n = 6) was only ~50% (P < 0.0001, Student's t test) of control (ΔI_{SC}: -9.8 ± 1.4 μA cm^{-2}, n = 14).

Insulin normally activated both PKB and SGK1 and, although Akt-I1/2 (1 μM) reduced the activation of PKB, this response was still seen in Akt-I1/2-treated cells (n = 9). Suprisingly, Akt-I1/2 also reduced the activation of SGK1 (n = 8). Experiments that explored the effects of higher concentrations of Akt-I1/2 (3 μM and 10 μM) revealed essentially complete inhibition of PKB at 10 μM (n = 6). However, at this concentration, Akt-I1/2 also prevented the activation of SGK1.

Although Akt-I1/2 inhibits the insulin-induced increase in I_{SC}, this finding cannot be cited in support of the idea that PKB is central to the control of Na^+ transport (Lee *et al.*, 2007) since Akt-I1/2 does not inhibit PKB selectively under the present conditions.

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MKM is supported by the MRC doctoral training fund.

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PC6

Glucocorticoid-inducible leucine ziper proteins (GILZ1 – 3) supress the dexamethasone-induced activation of the a-ENaC gene promoter

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Studies of renal epithelial cells have shown that glucocorticoid-inducible leucine zipper proteins (GILZ1-3) contribute to the hormonal control of epithelial Na^+ channel (ENaC) activity by suppressing signalling via the extracellular signal regulated kinases (ERK1/2) and by potentiating the effects of serum and glucocorticoid-inducible kinase 1 (SGK1) upon the abundance of ENaC in the membrane (Soundararajan et al., 2005, 2009). Moreover, recent work from this laboratory (Watt et al. 2009) has shown that transient expression of GILZ1 mimics the effects of glucocorticoid stimulation by activating an endogenous, Na^+-permeable conductance in hormone-deprived H441 human airway epithelial cells. Since glucocorticoids are also known to control the α-ENaC gene transcription via a mechanism that is supressed by ERK1/2 (Wang et al., 2000), the present study has explored the effects of GILZ1-3 upon the dexamethasone-induced activation of a reporter gene construct incorporating 2.2 kb upstream sequence of the α-ENaC gene promoter.

Initial studies showed that U1026 (10 μM), an inhibitor of ERK1/2, enhanced that response to maximally effective concentrations of dexamethasone (Fig 1A), a result which accords well with earlier work (Wang et al. 2000). Although the EC_{50} values for dexamethasone measured in cells expressing GILZ1-3 (GILZ1: 4.5 ± 0.6 μM; GILZ2: 4.8 ± 0.7 μM; GILZ3: 1.9 ± 1.6 μM) did not differ significantly from the corresponding control values (4.8 ± 0.4 μM; 4.2 ± 0.6 μM and 2.1 ± 0.2 μM respectively), each of these proteins caused clear inhibition of the

responses to maximally-effective concentrations of dexamethasone (Fig 1B-C).

Transient expression of GILZ1-3 therefore supress α-ENaC gene transcription via a mechanism that cannot be attributed to inhibition of ERK1/2. It is therefore possible that these proteins may permit negative feedback control over the expression of this gene in glucocorticoid-stimulated cells.

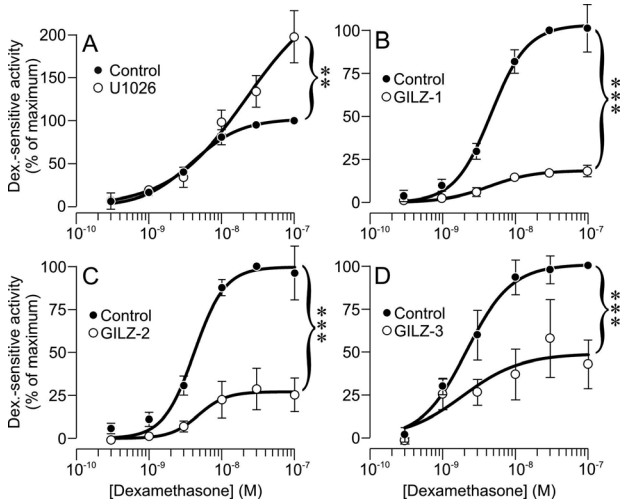

Fig 1. H441 airway epithelial cells expressing a luciferase linked reporter gene construct incorporating the α-ENaC gene promoter were exposed to dexameathasone (0.3 - 100 nM) for 18 h before luciferase formation was quantified. Experiments were undertaken using strictly paired protocols in which control and experimental cells were age matched and at identical passage, and all transcriptional activities (mean ± s.e.m) have been normalised to the activity measured in control cells exposed to a maximally effective concentration of dexamethasone. Sigmoid curves were fitted to the experimental data by least squares regression, and the astesixes denote statistically significant effects upon the transcriptional activities evoked by maximally effective dexamethasone concentrations (Student's t test, ** P < 0.01; *** P < 0.001). A. Effects of U1026 (10 μM, n = 5). B - C Effects of transiently expressing GILZ1 - 3 (n = 5 for each).

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PC7

Does CFTR play a role in regulating cation permeable channels in airway epithelia?

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP/protein kinase A regulated chloride (Cl^-) channel localised to the apical membrane of epithelial cells. It plays an important role in maintaining salt and fluid balance in these cells. As well as an ion channel CFTR has been found to regulate a number of transport proteins, e.g. the epithelial sodium channel ENaC. In cystic fibrosis (CF) which is caused by mutations in CFTR there is abnormal calcium (Ca^{2+}) homeostasis in airway epithelial cells. Given the role CFTR plays as a channel regulator, cation currents in normal and CF airway epithelial cells were examined. Whole cell ruthenium red (RR, 100 μM) and cadmium (Cd^{2+}, 100 μM) sensitive conductances were measured in 16HBE14o- and CFBE41o- cells under control conditions or after cAMP stimulation. The bath solution contained NaCl and the pipette solution contained CsAsp. A ramp protocol measured currents between +60 to -100mV. Statistical significance was tested using Students t-test and assumed at the 5% level. Under control conditions both 16HBE14o- and CFBE41o- cells possessed a RR and Cd^{2+}- sensitive conductance. Cd^{2+} caused a significant reduction in the size of the RR-sensitive conductance compared to RR on its own in 16HBE14o- cells (397.8 ± 77.9μS/cm^2, n=8 vs 881 ± 187.6μS/cm^2, n=9 respectively) but not in CFBE41o- cells (414.4 ± 74.7μS/cm^2, n=10 vs 460.9 ± 112.4μS/cm^{2+}, n=7 respectively). Under cAMP stimulated conditions both 16HBE14o- and CFBE41o- cells possessed a RR-sensitive conductance. After cAMP stimulation 16HBE14o- cells failed to show a Cd^{2+}- sensitive conductance, while CFBE41o- cells possessed a small Cd^{2+}-sensitive conductance (184.1 ± 38.5μS/cm^2, n=9). In stimulated 16HBE14o- and CFBE41o- cells Cd^{2+} had no effect on the size of the RR-sensitive conductance compared to RR on its own (428.5 ± 47.8μS/cm^2, n=13 vs 542.5 ± 132.8μS/cm^2, n=11 in 16HBE14o- cells and 613.2 ± 143.3μS/cm^2, n=12 vs 567.4 ± 112.3μS/cm^2, n=13 in CFBE41o- cells). Consistent with previously found selectivity differences, these data suggest that different cation channels are found in normal and CF airway epithelial cells. Further studies are needed to confirm whether these differences are a consequence of mutations in CFTR.

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Dr Louise Robson and Dr Richmond Muimo. This work was supported by a University of Sheffield Harry Worthington Scholarship.

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PC8

Activation of PKC causes a reduction in lung liquid absorption rate in adult rat lungs

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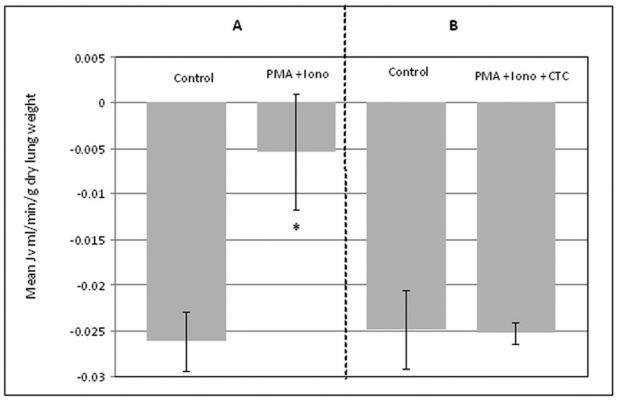
Previous alveolar epithelial cell culture studies have shown that both the amiloride-sensitive epithelial sodium channel (ENaC) activity and its mRNA can be down regulated by protein kinase C (PKC) (1) The aim of this work was to determine whether or not PKC activation can reduce the net lung liquid absorption rate (J_v) in adult rat lungs.

Male Wistar rats were anaesthetised using a solution of 25% Hypnorm (Fentanyl Citrate & Fluasinone), 25% Hypnovel (Midazolam) and 50% water (2.7 ml/kg i.p). The rat was ventilated

with room air then a median sternotomy was performed and the pulmonary artery and left atrium were catheterised. The lungs were perfused with a modified Ringer's solution containing 3% albumin. 15ml/kg body weight of liquid, containing an impermanent tracer (Blue Dextran), was instilled into the lung lumen. The lung liquid (LL) volume was calculated by measuring absorbance of LL samples at 620nm. LL volume was plotted against time to calculate J_v.

The PKC activator phorbol-12-myristate-13-acetate (PMA 0.1μM) was instilled into the LL after a control period. This resulted in no significant change in the J_v (p= 0.87). However, when a calcium ionophore, ionomycin (1μM) was given in conjunction with PMA, Jv was reduced from -0.0262 ± 0.003 ml/min/g dry lung weight (mean ± S.E.M.) to -0.005 ± 0.006 ml/min/g dry lung weight (p= 0.03; Figure 1).

The reduction in J_v caused by PMA and ionomycin was blocked by the PKC inhibitor chelerythrine chloride (1μM), -0.025 ± 0.004 ml/min/g dry lung weight to -0.025 ± 0.001 ml/min/g dry lung weight (p= 0.933; Figure 1). This result was confirmed when a different PKC inhibitor GF109203X was used (0.1μM) p= 0.521. From results obtained it appears that PKC does indeed regulate lung liquid absorption. The use of a PMA in conjunction with ionomycin suggests that the PMA is acting on the calcium dependent isoenzyme (c)PKC. This reduction in J_v was blocked using two separate PKC inhibitors suggesting that the mechanism is most likely PKC dependant.



(A) Effect of PMA and ionomycin (Iono) on mean resting J_v *P = 0.03 compared to control using paired t-test. (B) Effect of PKC inhibitor, chelerythrine chloride (CTC) on the PMA and ionomycin reduction of J_v.

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PC9

Intestinal ciprofloxacin secretion is mediated by multiple ABC transporters

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Fluoroquinolones such as ciprofloxacin are secreted into the intestinal lumen and this is an important route for whole body drug elimination. Our own work has shown that ciprofloxacin is not secreted by MDR1-transfected MDCKII epithelial monolayers nor is it subject to inhibition by MRP inhibitors such as MK-571 (Lowes and Simmons, 2002). Recently intestinal secretion via BCRP has been suggested as the major route for intestinal secretion (Merino et al, 2006). The purpose of the present study was therefore to evaluate the role of BCRP in secretion and to investigate whether secretion in the human intestinal cell model, Caco-2, is mediated solely by BCRP.

Epithelial monolayers of human Caco-2 cells native MDCKII cells, murine bcrp-MDCKII cells and human BCRP-MDCKII cells were grown on Transwell polycarbonate filters after high-density seeding (Lowes and Simmons, 2002) and transepithelial fluxes in the apical to basal (Ja-b) and basal to apical (Jb-a) surfaces determined using ^{14}C-ciprofloxacin (total concentration 10μM). Whereas in native MDCK layers no net secretion of ciprofloxacin was evident (Ja-b = 0.174 ± 0.019 nmol/cm^2/hr, Jb-a = 0.127 ± 0.014 nmol/cm^2//hr, N = 3 experiments of 3 replicates, means ± S.E.M.), a significant net secretion was observed in mbcrp-MDCK II epithelia (Jnet= Jb-a – Ja-b, 0.187 ± 0.02 nmol/cm^2//hr N = 3, p<0.01, single tailed T test vs 0). In contrast, hBCRP-MDCKII epithelial layers showed no net secretion (Jnet= -0.07 ± 0.06 nmol/cm^2//hr, N = 3). Ciprofloxacin secretion by mbcrp-MDCKII cell-layers was inhibited by a specific BCRP inhibitor (1μM Ko143, Allen et al 2002) from control values of Jnet of 0.187 ± 0.01nmol/cm^2//hr, n= 3 to 0.013 ± 0.012 nmol/cm^2//hr, n= 3, p<0.01, unpaired means T test). For 2 strains of Caco-2 cells, ciprofloxacin secretion was observed (Jnet= 0.39 ± 0.07 nmol/cm^2//hr, n= 3 for both) but secretion was only partially inhibited by 1 μM Ko143 by 10 % and 53% respectively. This data is therefore consistent with both BCRP mediated and BCRP-independent transport pathways. In order to test whether MRP4 may mediate ciprofloxacin secretion, cellular accumulation of ^{14}C-ciprofloxacin (total concentration 10μM) by MRP4 transfected HEK cells was determined. Ciprofloxacin accumulation was reduced by MRP4 transfection consistent with ciprofloxacin being an MRP4 substrate. However, though MK571 was an effective MRP4 inhibitor in MRP4-HEK cells, it did not inhibit transepithelial ciprofloxacin secretion by Caco-2 cells. We conclude that ciprofloxacin is a substrate of for both BCRP and MRP4 but that the substantial fraction of ciprofloxacin secretion by Caco-2 epithelia is mediated by neither BCRP nor MRP4

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PC10

Compound C increased AMPK activity and inhibition of amiloride-sensitive Na^+ transport induced by glucose deprivation in human H441 airway epithelial cells

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We previously showed that pharmacological activation of adenosine monophosphate activated protein kinase (AMPK) inhibited Na+ transport in H441 human airway epithelial cells. However, the effect of physiological activators has not been investigated. Removal of glucose from the medium (glucose deprivation; < 0.4 mM) for 4 hours increased phospho/total acetyl CoA carboxylase (ACC) (a target for AMPK mediated phosphorylation) as analysed by western blotting from 0.5 ± 0.06 to 0.9 ± 0.13 densitometry units, P = 0.04, n = 4 in H441 cells. In addition, phospho/total AMPK was correspondingly increased. Glucose deprivation significantly decreased spontaneous short circuit current across H441 monolayers to 80.8 ± 5.30 % (P = 0.008, n = 8 of the controls), without any significant effect on the resistance of the monolayers. Glucose deprivation also significantly reduced amiloride-sensitive transepithelial Na+ transport from 29.1 ± 2.56 to 21.6 ± 0.19 μA/cm2, P < 0.009, n = 8. In the presence of Compound C (80 μM), a pharmacological inhibitor of AMPK, amiloride-sensitive transepithelial Na+ transport was reduced from 23.2 ± 2.67 to 6.9 ± 0.75 μA/cm2 by glucose deprivation. It was significantly lower than monolayers deprived of glucose alone (P < 0.009, n = 8). Interestingly, Western blotting demonstrated that Compound C further increased phospho/total ACC induced by glucose deprivation significantly from 1.3 ± 0.12 to 2.7 ± 0.23 densitometry units, P = 0.001, n = 4. Phospho/total AMPK was also correspondingly increased. These data indicate that glucose deprivation increased AMPK activity and this correlated with decreased ion transport in H441 cell monolayers. However, that Compound C augmented the effect of glucose deprivation indicates that care must be taken when interpreting the results obtained from its use. Supported by the BBSRC and St George's University of London.

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PC11

Copper loading and depletion regulate iron transporter expression in Caco-2 cells

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Copper is an essential trace metal in the body required for many physiological functions. The intimate relationship between copper and iron metabolism has been known for many years (Sharp, 2004). In in vitro intestinal epithelial cell models, experimental evidence suggests that copper and iron may compete for uptake via divalent metal transporter 1 (DMT1) (Tennant et al 2002). Furthermore, in the same cells hephaestin, a copper-containing ferrioxidase, is required for iron efflux (Vulpe et al 1999). The aim of this study was to investigate the effects of copper on the protein and mRNA expression of the iron transporters DMT1 and ferroportin, and the ferrioxidase hephaestin in intestinal Caco-2 cells.

Fully-differentiated Caco-2 cells (used for experiments 21 days post-seeding) were treated with 50 μM copper chloride, for 4, 8, and 24 hours. In addition, the effect of treating cells with 0.5 mM of Triethylenetetramine dihydrochloride (TETA), a copper chelator, over the same time scale was investigated. We examined the changes in the whole cell levels of transport proteins by western blotting and mRNA expression by Real Time PCR. Western blotting data were semi-quantified using Scion Image software for were analysed by one-way ANOVA and Tukey's post hoc test (significant at $p<0.05$).

Following exposure to copper for 24h there was a significant decrease in DMT1 mRNA expression (-42%; $p< 0.05$) compared with control. Ferroportin and hephaestin were not altered under copper-loading conditions. Treatment with TETA (0.5mM) for 24h, significantly increased DMT1 (+380%; $p<0.006$) and hephaestin mRNA expression (+1420%; $p<0.04$). In addition, there was a significant increase in hephaestin protein expression (+183%; $p<0.05$) in TETA treated cells.

These data show that the expression of iron transporters is altered by modulating cellular copper levels and provide further evidence that copper nutrition exerts an important influence on iron homeostasis.

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PC12

Selenium intake, antioxidant protection and inflammatory signalling

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Selenium is a micronutrient essential for human health. Selenium supplementation has been reported to lower mortality from colon cancer[1, 2]. Selenium is present in selenoproteins including glutathione peroxidases (GPx) 1-4. GPxs are antioxidant enzymes that protect cells from excessive oxidative stress. Knock-out of GPx1 and 2 leads to increased sensitivity to bacterial-induced inflammation in the gut suggesting they may have a role in maintaining innate immunity homeostasis against inflammatory stimulation from exposure to luminal bacteria.

GPx4 has been linked to leukotriene metabolism [3, 4]. The aim of the present work was to examine the role of selenium and GPxs in the gastrointestinal cell line Caco-2 in relation to inflammatory stimulation.
To assess inflammatory signalling, a luciferase reporter cell model was developed in Caco-2 cells, in which luciferase expression was under the control regulatory elements that bound the Nuclear Factor-kappa B, the core inflammatory transcription factor[5]. As a control reporter luciferase coding sequences were linked to a TATA box. Using cells expressing these constructs the impact of GPx4 knockdown on Nuclear Factor-kappa B activation by endogenous (TNFα) and exogenous(bacterial flagellin) inflammatory stimuli was investigated. Using small interfering RNA GPx4 expression was knocked down by approximately 80% as assessed by RTPCR and Western blotting. Knock-down had no effect on activation of luciferase activity by either stimulus. However, when cells were grown in Se deficient conditions a 30% increase in TNFα-induced reporter activity was observed. Future work will address how other GPxs and selenium modulate Nuclear Factor-kappa B signaling in intestinal epithelial cells (and thus protect gut cells from inflammation) and the possible importance of radical oxygen species in this mechanism.

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PC13

Effect of Lipoxin A_4 in modifying the airway surface liquid layer

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A key aspect of the lung innate defence system is the ability of the epithelium to regulate the Airway Surface Liquid (ASL) volume. The ASL electrolyte composition, volume and height are tightly regulated by transepithelial ion and water transport. Regulation of ASL physiology is required for effective ciliary beat and muco-ciliary clearance in the proximal airways. The net transepithelial Na+ absorption and Cl- secretion are closely controlled to maintain an appropriate ASL layer on the bronchial airway surface through the activity, respectively, of the amiloride-sensitive epithelial sodium channel (ENaC) and the Cystic Fibrosis Transmembrane conductance Regulator. Lipoxin A4 (LXA_4) is an endogenous anti-inflammatory molecule produced from arachidonic acid that has been reported to be reduced in inflammatory Cystic Fibrosis (CF) lung[1].
CF is a severe genetic disease caused by mutations in the CFTR gene, which result in a marked reduction in Cl- secretion and an increased Na+ absorption. This electrolyte imbalance alters the ASL homeostasis and leads to a dehydrated airway lumen. Dehydration limits mucociliary clearance and favours chronic bacterial infection and inflammation. One of the therapeutic avenues in CF is to restore the depleted ASL by correcting the ion transport defects. We have investigated the effect of LXA_4 on the ASL height in CF and non-CF cell lines, and the possible role of LXA_4 in inhibiting Na+ hyper-absorption.
Materials and Methods: CF (CuFi-1) and non-CF (NuLi-1) cell lines were grown to confluency on semi-permeable filters in order to obtain a differentiated polarised epithelium showing high transepithelial resistance and loaded with fluorescent dye Calcein AM (5μM). Dextran solution containing the Texas Red fluorochrome was applied on the apical side of the epithelium in order to label the ASL compartment. Live cell ASL height was measured using a laser scanning confocal microscope.
Results: The steady-state ASL height in the CF epithelium was reduced when compared to the normal non-CF epithelium. The addition of LXA_4 (1nM) for 15 minutes to the basolateral side significantly increased ASL height from 5±0.28 μm (n=18) to 15.25±1.18 μm (n=19) in CuFi cells and from 9±0.27 μm (n=19) to 14.26 ±0.67 μm (n=46) in NuLi cells. The effect of LXA_4 to increase ASL height in CuFi cells was observed to be sustained over time to a level of 9.23±0.56 μm (n=28) at 30 min, and 7.86±0.92 μm (n=10) at 45 minutes (n=9). These rapid and sustained effects of LXA_4 on ASL height were abolished by the lipoxin receptor antagonist, boc2 (10nM).
Discussion: LXA_4 treatment resulted in an increased ASL height in a CF bronchial epithelium cell line and may provide a novel avenue in complementing existing therapy.

Karp CL et al. Cystic fibrosis and lipoxins. Prostaglandins Leukot Essent Fatty Acids. 2005; 73(3-4):263-70

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PC14

Inhibition of the glycine transporter GLYT1 blocks glycine protection of intestinal cells

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Glycine is cytoprotective against a range of toxic challenges, particularly, in intestine, against ischemia-reperfusion injury and oxidative stress (Schaefer et al, 2008; Kallakuri et al, 2003;

Lee et al, 2002). To investigate the mechanism of cytoprotection in intestine we used the model cell lines Caco-2 and HCT-8, representative of enterocytes and colonocytes respectively, where GLYT1 activity accounts for 30-40% of basolateral glycine uptake (Christie et al, 2001; Tahir et al, 2005). Cells grown on 96-well plates were exposed to the oxidising agent tert-butylhydroperoxide (t-BOOH) which induced formation of reactive oxygen species (ROS) and a reduction in cell viability (viability following 150μmol L^{-1} t-BOOH, 28 ± 2 (mean ± S.E.M.)% of control cells; n = 12, p< 0.05 (ANOVA with Dunnett's post-test)) and intracellular glutathione concentration (concentration following 100μmol L^{-1} t-BOOH, 77.5 ± 1.8 % of control, n = 12, p <0.05). Pre-treatment with glycine (1 or 5 mmol L^{-1}) was protective against t-BOOH challenge and increased cell viability (63 ± 7 and 71.5 ± 3.3% of control respectively, p<0.05 compared to t-BOOH treated cells), reduced induced ROS concentration (75.9 ± 2.17 and 77.02 ± 4.34 % of t-BOOH treated respectively, p<0.05) and increased glutathione levels (concentration in 5mmol L^{-1} glycine pretreated cells: 99.6 ± 3.2% of control, p<0.01 compared to t-BOOH treated). Glycine given concurrent with or after oxidative challenge had no protective effect. Inhibition of GLYT1 activity with the specific inhibitor ALX-5407 (N [3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)-propyl] sarcosine (NFPS)) reduced Na^+ -dependent, alanine insensitive glycine uptake by ~75%, whereas the GLYT2 inhibitor ALX-1393 (O-[(2-Benzyloxyphenyl-3-flurophenyl)methyl]-L-serine) had no effect, consistent with data demonstrating lack of GLYT2 expression in these cell lines. When cells were incubated with ALX-5407 prior to glycine treatment and subsequent oxidative challenge, the protective effects of glycine were blocked: cell viability (t-BOOH treated: 53.3 ± 5.2 % of control; 5mmol L^{-1} glycine pre-treated: 48.3 ± 1.7 % of control, p>0.05 compared to t-BOOH treated) and glutathione concentration (72.2 ± 4.42 % of control, p< 0.05) were reduced compared to controls and ROS concentration was comparable to that in cells treated with t-BOOH alone (105.9 ± 3.83 and 102.1 ± 4.65% for 1 and 5mmol L^{-1} glycine pre-treated cells respectively, p>0.05). We conclude that glycine acquired across the basolateral membrane of intestinal cells by GLYT1 is used in cell defence and in maintaining intracellular antioxidant potential.

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

PC15

Immunoreactive ceramide is increased and associated with neutrophilic inflammation in the lower airway epithelium of people with cystic fibrosis

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Introduction and Objectives: Cystic fibrosis (CF) results from alterations in the CF transmembrane conductance regulator (CFTR) gene but the exact pathogenesis of lung disease remains poorly understood. Ceramide is an essential constituent of plasma membranes and regulates many cellular responses. It has recently been shown that CFTR-deficient mice accumulate ceramide in airway epithelial cells resulting in inflammation and susceptibility to *Pseudomonas aeruginosa* – two key features of CF lung disease.[1] Ceramide accumulation was also demonstrated in nasal epithelial cells from people with CF and qualitatively in a small number of lower airway sections.[1] The objective of this work was to evaluate ceramide levels quantitatively in the lower airway epithelium of people with CF compared to pulmonary hypertension (PH), emphysema and unused donors and to investigate relationships between epithelial ceramide and markers of neutrophilic inflammation and *P. aeruginosa* infection.

Methods: Immunohistochemistry was performed on airways from explanted lungs (8 CF, emphysema and PH respectively) and 8 donor lungs using ceramide (Glycobiotech), neutrophil elastase (NE) and myeloperoxidase (MPO) antibodies. Ceramide staining was evaluated in the lower airway epithelium in a blinded fashion using image analysis software and expressed as percentage area. A mean from 5 randomly selected, non-overlapping high-power fields was used for each patient. The number of cells staining positive in the airway for NE and MPO/millimetre basement membrane was also evaluated. Differences were compared using the Mann-Whitney test.

Results: Staining for ceramide was significantly increased in the lower airway epithelium of people with CF (median 14.11%, range 8.32-16.2) compared to PH (3.03%, p=0.0009, 2.05-5.72), unused lung donors (3.44%, p=0.0009, 1.86-4.9) and emphysema (5.06%, p=0.01, 1.52-13.24) (Figure 1). Ceramide staining was increased in emphysematous lungs compared to PH (p=0.0135) and unused donors (p=0.0009). The number of NE and MPO positive cells in the airway was positively correlated with the percentage of epithelium staining for ceramide (Pearson correlations 0.634, p<0.000 and 0.704, p<0.000 respectively). Ceramide staining was significantly increased in lungs colonised with *P. aeruginosa* (median 10.1%, range 1.52-16.2) compared to those not colonised (3.14% p=0.0106, 2.05-10.6).

Conclusions: Ceramide accumulates in the lower airway epithelium of people with CF, and to a lesser extent in emphysema, and is positively correlated with markers of neutrophilic inflammation and *P. aeruginosa* infection. This data supports the hypothesis that ceramide plays a role in the pathogenesis of CF lung disease and may represent a target for pharmacotherapy.

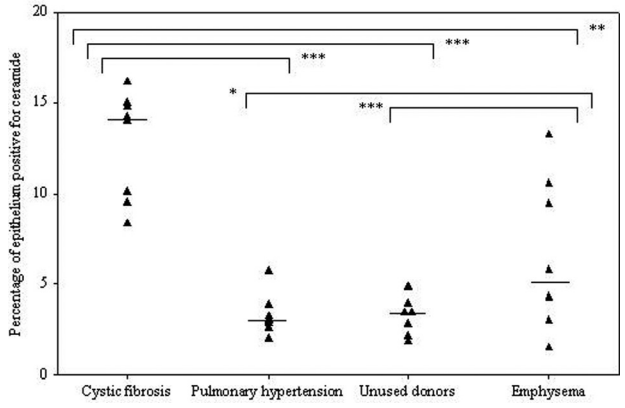

Figure 1: Results of ceramide staining, each symbol represents an individual patient and is the mean of the percentage epithelium staining positive in five randomly selected high power fields. The horizontal bar represents the median for each group. (*p=0.0135, **p=0.01, ***p=0.0009)

Teichgraber *et al.* (2008) *Nature medicine* **14**,382-391

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PC16

Responses of the COBW domain-containing genes to zinc in human Caco-2 intestinal epithelial cells and effects of zinc on the COBW domain-containing protein

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We identified the 6 human paralogous COBW domain-containing *(CBDW)* genes as potentially responsive to zinc through an in silico search for an element (ZRE; Zinc Responsive Element) found to mediate transcriptional repression of the zinc transporter *SLC30A5* gene in Caco-2 cells in response to zinc. The function of the CBDW proteins is unknown. Our hypothesis was that *CBWD* genes respond to zinc in Caco-2 cells and that CBDW protein expression is affected by zinc.

All data are stated as mean ± S.E.M in arbitrary units and statistical analysis was by Student's unpaired t-test. The abundance of the *CBWD* transcript in Caco-2 cells, normalised to *GAPDH*, was measured by RT-qPCR to be ~50% lower (P<0.001) in cells treated for 24 h with 100 μM zinc (0.48 ± 0.06, n=6) compared with 3 μM zinc (1.00 ± 0.09, n=6).

Zinc-regulated *CBDW* transcription and the role of the ZRE was investigated using plasmid constructs comprising the region -1078 to +98 of the *CBDW3* gene, relative to the start of transcription, upstream of the β-galactosidase reporter gene in the vector pBlueTOPO (Invitrogen). Reporter gene expression was lower (P<0.001) at 100 μM zinc (0.73 ± 0.04, n=23) compared with 3 μM zinc (1.00 ± 0.04; n=24) in transfected Caco-2 cells treated with zinc for 24 h. The response of the reporter gene to zinc was attenuated (P<0.01) by mutating the ZRE to a random sequence (1.00 ± 0.03 at 3 μM zinc; 0.90 ± 0.02 at 100 μM zinc; n=12), consistent with the ZRE having a role in mediating the transcriptional response to zinc.

Human CBWD3 protein, incorporating a C-terminal FLAG tag, was expressed transiently from a plasmid construct comprising the full ORF of the human *CBDW3* gene in the vector pCMV6Entry (Origene) in CHO cells treated for 24 h with 3 or 100 μM zinc. Western blot analysis using an anti-FLAG antibody indicated greater abundance (P<0.05) of the recombinant protein resolved by SDS-PAGE at the expected molecular weight (~44 kDa) in cells cultured at the higher zinc concentration (1.00 ± 0.12 at 3 μM zinc; 2.57 ± 0.36 at 100 μM zinc; derived by densitometric quantification of band intensity, n=3-4). Preliminary observations indicated increased abundance at the lower zinc concentration of an anti-FLAG immunoreactive band of a lower molecular weight (~30 kDa), possibly indicative of zinc-dependent cleavage.

These observed effects of zinc availability on the expression of the *CBDW* genes and CBDW protein are consistent with the view that the human CBDW proteins play a role in zinc homeostasis in intestinal epithelial cells. Further studies, including the identification of CBDW protein binding partners, description of tissue and sub-cellular distribution, and effects of zinc and other divalent metals on these measures, may help to elucidate this role.

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PC17

Comparison of P2Y receptor subtypes in equine sweat gland epithelial cells from normal and anhidrotic animals

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P2Y receptors are involved in epithelial transport in various tissue types (1), including sweat gland secretion via an increase in chloride permeability and changes in intracellular calcium ($[Ca2+]_i$) (2). Epithelial cells from isolated sweat glands of normal horses increase $[Ca2+]_i$ in response to purinergic agonists such as ATP and UTP (3). Anhidrosis, or inability to sweat, is a debilitating condition affecting humans and horses thought to be caused by a failure in the secretory process. The aim of this study was to compare the presence and responses of P2Y receptor subtypes in sweat gland epithelial cells obtained from anhidrotic horses with those from normal animals.

Epithelial cell lines were obtained from horse skin samples of normal, intermediate- and late-anhidrotic racing thoroughbreds in Hong Kong with ethics and owner approval. Local anaesthetic Lignocaine was injected into the skin prior to biopsy. Cells were cultured using standard tissue culture techniques. Epithelial cell lysates were used for Western blotting using antibodies against $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Cells were examined for changes in $[Ca2+]_i$ evoked by various purinergic agonists using calcium imaging techniques. Values presented as mean ratio units ± S.D. Statistical analyses were carried out

using one-way ANOVA and Bonferroni multiple comparison test, where P<0.001 was considered significant.
Western blot analysis showed bands for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in all cell types. Higher band density was observed in late-anhidrotic cells for $P2Y_2$ and $P2Y_4$ receptors than in either intermediate-anhidrotic or normal cells. ATP (100μM) and UTP (100μM) evoked significantly higher $[Ca2+]_i$ increases in intermediate-and late- anhidrotic cells in comparison to normal cells (Table 1). Additionally, UTP elicited a significantly higher $[Ca2+]_i$ response than ATP in intermediate- and late-anhidrotic cells, but not in normal cells (Table 1). ADP (100μM, n=18) and UDP (100μM, n=18) elicited no response from any cell type.
The results indicate that there may be additional $P2Y_2$ and $P2Y_4$ receptors in anhidrosis, which could account for the increased $[Ca2+]_i$ response to UTP, as both of these receptors are sensitive to UTP. $P2Y_6$ (UDP-sensitive) receptors were also found to be present in all cell types, but UDP failed to increase $[Ca2+]_i$, possibly demonstrating non-functional $P2Y_6$ receptors in these cells. These findings suggest a potential role for P2Y receptors in anhidrosis.

Table 1. $[Ca2+]_i$ responses to ATP & UTP

	ATP ($[Ca2+]_i$ ratio units)	UTP ($[Ca2+]_i$ ratio units)
Normal	0.2±0.1 (n=54)	0.2±0.1 (n=54)
Intermediate-anhidrotic	0.5±0.1 (n=54)*	0.7±0.2 (n=54)*†
Late-anhidrotic	0.5±0.1 (n=54)*	0.7±0.1 (n=54)*†

(* significantly higher than normal, P<0.001; † significantly higher than ATP, P<0.001)

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PC18

Inhibition of A20 signalling in cystic fibrosis epithelium

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Introduction: The zinc finger protein A20 is responsible for the termination of NF-κB signalling through immune receptors including Toll-like receptors (TLRs). A20 deficient mice suffer from persistent activation of NF-κB by TLRs and tumour necrosis factor receptor, resulting in mortality among neonates and multiorgan inflammation in adults [1]. A20 has been shown to be a negative regulator of TLR2 and TLR4 induced inflammatory cytokine (IL-8) release in the airway epithelium [2] and, more recently, was found to attentuate allergic asthma in mice [3]. Cystic Fibrosis (CF) is characterised by chronically inflammed lung disease, which is the primary cause of mortality amoung sufferers. We hypothesise that in the CF epithelium, defective A20 action leads to enhanced pro-inflammatory signalling through the NF-κB pathway.
Methods: Non-CF (HBE) and CF (CFBE) bronchial epithelial cell lines were grown to 80% confluency on collagen-coated plates prior to stimulation with 50μg/ml *PA*-lipopolysaccharide (LPS; Sigma). A20 and NF-κB (P50 and P65) levels were measured by flow cytometry, 0-12h after stimulation. Nuclear extracts for NF-κB staining were obtained using a DNA CycleTest kit (BD Biosciences). IL-8 release was measured by ELISA (Peprotech) using supernatants from cells treated with *PA*-LPS for 24h. Statistics were calculated by ANOVA, with n=3-6 for all experiments.
Results: IL-8 release was significantly higher in CFBE cells (p<0.05). Despite increased intracellular A20 expression 4h (P<0.001) after *PA*-LPS exposure, expression in CFBE cells fell below basal levels by 12h exposure, while HBE cells showed sustained A20 expression. Basal NF-κB (both P50 and P65) expression in CFBE cells was significantly greater than in HBE cells (P<0.0001). P50 levels remained unchanged in HBE cells following *PA*-LPS stimulation, while CFBE cells showed significant increases (2 and 4h after stimulation, P<0.001). P65 expression increased significantly in both HBE and CFBE cells with exposure to *PA*-LPS (P<0.05-0.001). However, these increases were more pronounced in CFBE cells. Notably, P65 expression remained significantly up-regulated at 12h (when A20 expression was inhibited) in CFBE cells and not in HBE cells, while P50 expression returned to basal levels in both cell types.
Conclusions: In CF epithelium, A20 signalling is significantly inhibited causing increased NF-κB activation, contributing to a state of chronic inflammation.

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PC19

The hydroxylase inhibitor DMOG attenuates colonic epithelial secretory function

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Gastrointestinal (GI) disorders, such as inflammatory bowel diseases and ischaemic colitis, cause inflammation of the GI tract and are commonly associated with hypoxia, altered epithelial transport and diarrhoea. In hypoxia, O_2 sensors known as prolyl hydroxylases (PHDs) are inactivated, thereby allowing transcription factors such as hypoxia inducible factor (HIF) and nuclear factor κB (NFκB) to be activated. While recent studies have shown that inhibition of PHDs is protective in murine colitis, little is known of their role in regulating intestinal epithelial transport function. Thus, the aim of the current studies was to investigate the role of hydroxylases in regulating intestinal epithelial secretory function.
The pan specific inhibitor dimethyloxalylglycine (DMOG) was used to inhibit hydroxylase activity. Cl^- secretion, the primary driving force for intestinal fluid secretion, was measured as

changes in short circuit current (I_{sc}) across voltage clamped monolayers of T_{84} cells. Results are expressed as mean ± SEM. Statistical analyses were made by one way ANOVA and Tukey multiple comparisons test. p values ≤0.05 were considered to be significant.

Pre-treatment of T_{84} cells with DMOG (1mM, 24 hrs) significantly attenuated Cl^- secretion in response to forskolin (FSK) and carbachol (CCh). Responses to CCh and FSK were 20.2% ± 2.6% (n=16; p≤0.001) and 38.6% ± 6.7% (n=11; p≤0.001) of those in control cells, respectively. The effects of DMOG on secretory responses were apparent after 3 hours and maximal by 18 hours, which was concurrent with DMOG-induced stabilisation of HIF1-α. Pre-treatment of T_{84} cells with $CoCl_2$ (500μM, 24hrs), which increases cellular HIF levels in a hydroxylase-independent manner, also attenuated Cl^- secretory responses to CCh and FSK to 51.6% ± 8.1% (n=3; p≤0.05) and 53% ± 8.4% (n=4; p≤0.01) of those in control cells, respectively. DMOG did not alter the generation of prosecretory second messengers in response to CCh and FSK. Similarly, secretagogue-induced basolateral K^+ and apical Cl^- conductances were unaltered by DMOG. In contrast, Na^+/K^+ATPase activity was significantly reduced (to 27.6% ± 8.1%; n=7 p≤0.01) by DMOG. Analysis of transport protein expression revealed that DMOG significantly decreased both protein and mRNA expression of CFTR and NKCC1 but did not alter expression of the Na^+/K^+ATPase α subunit. Finally, cellular ATP levels were significantly reduced (to 67% ± 8.5% of controls; n=6; p≤0.01) by treatment with DMOG.

These studies demonstrate a novel role for PHDs in regulating intestinal epithelial secretory function by a mechanism which appears to involve HIF, depletion of cellular ATP, and inhibition of transport protein function. Our data suggest that by virtue of their ability to alter epithelial transport, hydroxylases are likely to be important regulators of intestinal fluid transport in health and disease.

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PC20

Differentiation of secondary hepatocyte cell lines towards an *in vivo* hepatic phenotype

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Assessment for the potential of liver toxicity during drug development currently utilises primary hepatocytes and animal models; however, these are costly and present ethical issues. Secondary heptocytes, derived from hepatic carcinomas, do not retain all the features of hepatocytes *in vivo* such as expression of key metabolic enzymes and are of limited value. To offer an alternative to current models, this research has attempted to differentiate the secondary hepatic cell lines HepG2 and Huh7 to produce cells which more closely resemble the *in vivo* hepatocyte. Initially, adaptations of protocols for differentiation of stem cells into hepatocyte-like cells (**1-4**) were used; combinations of hepatocyte growth factor (HGF), fibroblast growth factor 4 (FGF4), and oncostatin M (OSM) along with dexamethasone, insulin-transferrin-selenium (ITS) and sodium pyruvate. Treatment times were limited to 4 days due to overgrowth and death of cells. To restrict growth, 1% di-methylsulfoxide (DMSO) was used (**5**) and cells were treated for up to 27 days. Finally, DMSO was combined with a selection of growth factors and applied for up to 25 days. RNA was extracted at regular intervals throughout the treatments, reverse transcribed, and abundance of specific mRNAs determined by real-time PCR. mRNA levels of genes associated with the differentiated hepatic phenotype such as albumin and transferrin, more abundant in mature than immature hepatocytes, were the focus of initial analysis. When treated with growth factors only, cells showed no clear indication of maturation, possibly due to the relatively short incubation time. Increased levels of albumin mRNA were seen with 1% DMSO in both HepG2 and Huh7 cells, following 15 and 9 days treatment respectively (HepG2, control = 133.1±26.2 (mean±S.E.M.), 15 days DMSO = 516.9±74.7, p<0.01. Huh7, control = 123.8±14.2, 9 days = 302.7±64.4, p<0.01 tested by 1-way ANOVA with Dunnett's post test, n=3. All data is given in arbitrary units.). HepG2 cells also showed significant increases in albumin and transferrin mRNAs after 5 to 10 days of treatment with HGF and 1% DMSO (albumin control = 133.1±26.2, 5 days = 308.2±38.8, 10 days = 686.0±61.1, p<0.01 for both. Transferrin control = 0.43±0.13, 5 days = 1.08±0.20, 10 days = 1.76±0.22, p<0.01 for both, n=6). Huh7 cells showed no signs of increased differentiation during growth factor plus DMSO treatment. These results indicate that with further refinement of treatment, a secondary hepatocyte may be altered to show a more differentiated hepatocyte phenotype.

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PC21

Expression and localisation of the pyrophosphate transporter, ANK, after vasopressin stimulation in murine collecting duct cells (mpkCCDcl4)

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We have previously identified expression of the pyrophosphate transporter ANK in murine kidney (Carr et al, 2007). ANK is expressed in the cortical collecting duct in both principal and intercalated cells. Since pyrophosphate is a potent inhibitor of calcium crystal formation and since nephrolithi-

asis is most likely to occur in concentrated supersaturated urine, we hypothesised that control of ANK protein expression and function may be controlled by vasopressin. Inactivating mutations in the gene for the mouse ANK protein not only increase calcium deposition in joints, causing arthritis, but mutant ANK mice also exhibit renal calcification (Ho et al, 2000).

RT-PCR confirmed ANKH expression in whole mouse kidney and two murine cortical collecting duct cell-lines, M1 murine epithelial cells and mpkCCDcl4 murine cortical collecting duct cells. mpkCCDcl4 cells were chosen for further study since they had previously been shown to respond to arginine vasopressin (AVP) treatment by increased expression of aquaporin 2 (AQP2) (Hasler et al, 2002). Confluent monolayers of mpkCCDcl4 cells were grown on Transwell filter inserts (Costar) in supplemented DMEM/F12 media for 6-10 days. By day 6 a significant transepithelial resistance (2.9 ± 0.3 kΩcm^2, n=140) and spontaneous p.d. (6-13 mV basal solution electropositive) were observed that was associated with transepithelial Na^+ absorption. Incubations with vasopressin (10nM) were made from the basal epithelial surface for 24 h. Monolayers were then fixed for immunocytochemistry by methanol immersion (0ºC 15 min) and stored at 4ºC prior to block with horse serum and then rabbit anti-ANKH antibody (1 in 250, Ab3, Ho et al, 2000) and goat anti-AQP2 (1 in 250, Santa-Cruz). Following donkey serum incubation as a secondary block cells were incubated with donkey anti-rabbit 488 (Alexa Fluor, Molecular Probes), or donkey anti-goat 568. In the absence of vasopressin, ANK was localised both at the lateral and apical plasma membranes and within cytoplasmic vesicles, AQP2 staining was negative. After vasopressin treatment, a proportion of cells (6.6%) showed marked up-regulation of AQP2 with strong apical staining. In addition a proportion (1.8%) showed a markedly increased expression of ANK at the apical plasma membrane. The AQP2 positive cell population was distinct from the apical ANK population. Similar data were obtained after 24h treatment with 10μM forskolin.

We conclude that ANK expression at the apical plasma membrane of mpkCCDcl4 cells is regulated by vasopressin via a cAMP-dependent pathway. ANK mediated pyrophosphate delivery to urine may be increased during urinary concentration helping to inhibit calcium crystal formation.

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PC22

Antisense clc-5 treatment disrupts endosomal acidification in mouse distal convoluted tubule (mpkDCT) cells

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Mutations in CLCN5, encoding the voltage-dependent Cl^-/H^+antiporter, CLC-5, cause Dent's disease by impairment of endocytosis mainly in the proximal nephron. Dent's is characterized by low-MW proteinuria, hypercalciuria, and nephrolithiasis. ClC-5 is expressed in acidic endosomes in collecting duct cells (mIMCD-3) where clc-5 ablation results in defective endocytosis and accumulation of annexin A2 at the membrane (Carr et al, 2006). Annexin A2 is implicated in the regulated expression of apical membrane channels TRPV5/6 (Van de Graaf et al, 2003). We have used mpkDCT cells as an appropriate model of a Ca^{2+}-transporting cell-type since they express TRPV5/6 (Diepens et al, 2004) to investigate a possible role for CLC in control of endocytic membrane retrieval in DCT cells.

RT-PCR confirmed expression of clc-5 mRNA in mpkDCT cells. Protein expression of clc-5 and trpv5 was demonstrated by immunocytochemistry and confocal microscopy using PEP5E1 antibody and anti-trpv5 (Santa Cruz). Confocal microscopy of clc-5-NT-GFP transfected mpkDCT cells with a 2 minute incubation with TRITC conjugated wheat germ agglutinin (WGA) lectin as a membrane marker revealed an intracellular location for clc-5-NT-GFP. LysoTracker Red (acidic endosome marker) or cotransfection with clc-5-CT-RFP and endosomal marker pEGFP-Endo showed substantial colocalisation of clc-5 with these markers. Using green fluorescent protein (GFP) as a transfection marker, internalisation of WGA lectin over 1h, as an endocytic marker, was determined in control GFP transfected cells and in cells where endogenous clc-5 was disrupted through cotransfection with antisense clc-5 and GFP (Carr et al, 2006). Endocytosis of WGA lectin in cells transfected with antisense clc-5 (n=18) was reduced in comparison to control cells (n=22) (quantification performed in mid-cell xy confocal sections, internalisation measured as a proportion of whole cell area, expressed relative to mean control values 11.08 ± 3.55% vs. 100 ± 15.18% respectively, $p<0.0001$). In addition, antisense clc-5 transfection also led to a marked reduction in intracellular acidic compartments labelled with LysoTracker Red (75nM, 30 min at 37ºC, Molecular Probes). Analysis of transfectants (quantification performed in mid-cell xy confocal sections as for lectin) revealed a reduction in the relative area of acidic compartments in antisense clc-5 (n=17) transfected cells when compared to GFP (n=22) transfected cells (30.99 ± 5.84% and 100.00 ± 13.89 % respectively, $p<0.001$).

Antisense clc-5 treatment results in an acidification defect in endosomes that interrupts the endocytic retrieval of plasma membrane in mpkDCT cells similar to proximal tubule. Defects in CLC-family proteins may result in dysregulation of apical membrane TRPV5/6 proteins within the distal nephron.

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PC23

Characterisation of urate secretion across human renal proximal tubule cell monolayers

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Urate is a major metabolite of purine metabolism. Urate homeostasis is a complex balance between urate production and renal clearance with marked species differences in the renal handling of urate. To understand urate handling in man we have investigated the transport of urate across polarised monolayers of human proximal tubule cells.

Proximal tubule cells were isolated from normal human kidney and grown as monolayers on permeable filter supports for up to 12 days. Under these conditions, PTC monolayers remained differentiated and expressed a full complement of transport proteins including OCT2, OAT1, OAT3, MDR1, MRP4 and BCRP at the appropriate membrane. To characterise urate handling by human proximal tubule cell monolayers, steady state, unidirectional fluxes of [^{14}C]-urate (45μM) and [^{3}H]-mannitol (5μM) were measured in pair-matched monolayers. In addition at the end of the flux period cell monolayers were excised and the intracellular accumulation of isotope was measured. Data are expressed as the mean ± sem of at least 4 monolayers per condition from a single kidney representative of 3 independent experiments. Statistical comparison was made using a one-way analysis of variance (ANOVA) test and a Dunnett post test.

We found a net secretion of urate across proximal tubule cell monolayers. The flux in the secretory direction (Jba) was significantly larger than the corresponding absorptive flux (Jab) of urate (7.7±0.3 v 2.7± 0.9 nmol/cm^2/h, n=4, P<0.01) generating a significant net secretion of urate across the monolayer (4.7±0.9 nmol/cm^2/h, n=4, P<0.001). Net secretion of urate was abolished in the presence of probenecid or PAH (both at 100μM) at the basolateral membrane. The OAT3-specific substrate estrone-3-sulfate inhibited net secretion by 42±3.2% (n=4, P<0.01) at the basolateral membrane. Net secretion was inhibited 87±6.1% (n=4, P<0.01) by the MRP2/MRP4 inhibitor MK571 (50μM) and by 21.2±7.8% in the presence of the BCRP inhibitor Fumetrimorgin-C (50μM) both at the apical membrane. Consistent with these observations, intracellular accumulation of urate within the monolayer was significantly less in the presence of either probenecid (19.3±3.9μM, P<0.01), PAH 11.4±1.8μM (P<0.01) or estrone-3-sulfate (28.3.±4.4μM, P<0.05) compared to control conditions (45.7±2.5μM). In contrast, inhibition of the apical exit step for urate by MK571 led to significant rise in intracellular urate concentration (82.3±5.6μM v 45.7±2.5μM, P<0.01).

Taken together, these data provide evidence for both cellular absorption and secretion of urate across proximal tubule cell monolayers. Urate flux in the secretory direction was significantly larger than in the absorptive direction. Urate secretion was mediated by both OAT1 and OAT3 at the basolateral membrane and predominately by MRP2/MRP4 at the apical membrane.

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PC24

Protein kinase D - a novel regulator of aldosterone-mediated ENaC expression and trafficking in renal cortical collecting duct cells

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Aldosterone treatment stimulates the phosphorylation and activation of Protein Kinase D 1 (PKD1) in murine renal cortical collecting duct cells (M1CCD), through the transactivation of the epidermal growth factor receptor (EGFR). PKD1 belongs to a family of serine/threonine kinases known to be important modulators of subcellular trafficking, through the regulation of vesicle fission from the Golgi. The epithelial sodium channel (ENaC) is a major effector of aldosterone action in the kidney and is crucial for the maintenance of whole body Na+ homeostasis. Fully active ENaC is believed to be a heterotrimer composed of one each of the alpha, beta and gamma subunits. ENaC activity is dynamically regulated by aldosterone through multiple mechanisms. The nuclear mineralocorticoid receptor (MR) in complex with aldosterone behaves as a ligand-dependent transcription factor which stimulates the tissue-specific upregulation of ENaC subunit expression. In the distal nephron, the ENaC alpha subunit is under the transcriptional control of ligand-bound MR, while beta and gamma are constitutively expressed.

Using M1CCD cells in which endogenous PKD1 was stably knocked down using siRNA, we examined the role that PKD1 plays in the aldosterone-mediated regulation of ENaC activity via transcription and/or trafficking of pre-expressed ENaC subunits, over an extended period of aldosterone treatment. The amiloride-sensitive, trans-epithelial current(ITE) was measured in wild-type (WT) and PKD1 suppressed M1CCD cells, grown to confluency on semi-permeable supports. A detectable rise in ITE was observed in WT cells within 2h of aldosterone treatment, an effect which was maximal after 24h. The induction of ITE by aldosterone was inhibited in PKD1 suppressed cells. Using immunocytochemistry and laser scanning confocal microscopy, we observed an increase in ENaC alpha expression in WT cells treated with aldosterone for 24h, an effect which was absent in PKD1 suppressed cells. Moreover, we observed an aldosterone-mediated induction of apical membrane insertion of the constitutively expressed ENaC beta subunit in WT cells.

Aldosterone treatment failed to affect the subcellular localization of ENaC beta in PKD1 suppressed cells. Overall, PKD1 plays a central role in the aldosterone-mediated regulation of ENaC activity, through both transcriptional control and subcellular trafficking.

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PC25

Active Cl^- and alveolar fluid transport under b-adrenergic stimulation, in the rat lung

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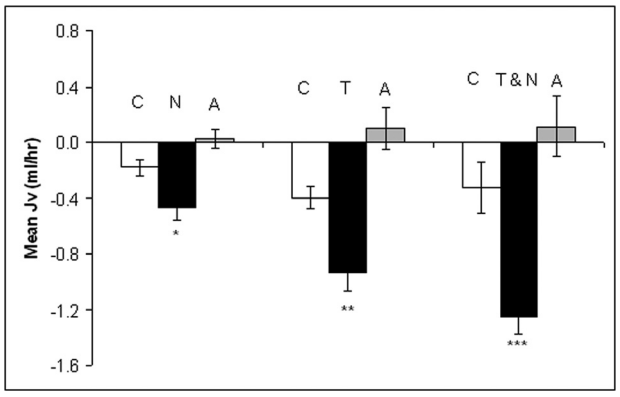
The pulmonary epithelium is lined by a thin layer of liquid, and as there is no net liquid transport at rest, there must be a dynamic balance between absorption and secretion. Active Na^+ transport underlies the resting absorptive process (1), and β-adrenergic stimulation is able to increase absorption of alveolar fluid markedly (2). Two mechanisms have been implicated in this process: the first involves direct stimulation of Na^+ conductance across the apical membrane of the epithelium (3); the second involves a role for active, transepithelial Cl^- transport, resulting in hyperpolarization of the apical membrane of the epithelial cell and thus an increased driving force for Na^+ uptake (4). Chloride channels with the characteristics of CFTR have been detected in alveolar type I and II cells, and the role of CFTR in the β-agonist-mediated alveolar fluid clearance was explored in studies with ΔF508 transgenic mice (5). The primary goal of this study was to test the hypothesis that the β-adrenergic stimulation of alveolar fluid clearance in the mature lung occurs as a result of activation of absorptive Cl^- channels. We performed experiments, using the in situ perfused rat lung. Male Wistar rats, aged 10-16 weeks, bred in house, were anaesthetized with a solution of fentanyl citrate with fluasinone and midazolam (2.7 $ml.kg^{-1}$ intraperitoneally) and ventilated. Following median sternotomy the pulmonary artery and left atrium were catheterized. The lungs were perfused in situ with a modified Ringer's solution containing 3% albumin. A known amount of an impermeant tracer (^{125}I albumin) was added to the lung instillate (LL) and from its change in concentration over time the rate of net transepithelial liquid movement (Jv) was calculated. Positive values of Jv indicate net secretion, negative values net absorption. The β2-agonist terbutaline (100μM into perfusate) induced an increase of 138.8% in the rate of absorption (from -0.39±0.08 to -0.94±0.12 $ml.hr^{-1}$, mean±SEM, p=0.05 by paired t-test, n=3) and the chloride channel blocker NPPB (100μM into the LL) induced an increase in the rate of absorption of 160% (from -0.18 ± 0.06 to -0.47±0.09 $ml.hr^{-1}$, p=0.03 by paired t-test, n=4) (Figure 1). Simultaneous administration of terbutaline and NPPB resulted in an increase in absorption of 281.2% (from -0.33±0.19 to -1.25±0.12 $ml.hr^{-1}$, p=0.005 by paired t-test, n=3) (Figure 1). Amiloride (100μM) given into the LL after NPPB or terbutaline caused the cessation of absorption.

These results suggest that the β-agonist stimulated alveolar fluid clearance is not mediated via activation of absorptive Cl^- channels, but rather via direct activation of amiloride-blockable Na^+ conductance. They also suggest the functional presence of secretory Cl^- channels in the alveolar epithelium, both in the resting state and under β-agonist stimulation.



The effect of blocking Cl^- and activating Na^+ conductance. C = control, N = NPPB, T = terbutaline and A = amiloride. *P = 0.03 (n = 4), **P = 0.05 (n = 3), ***P = 0.005 (n = 3); all compared to corresponding controls by paired t-test.

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PC26

Aldosterone regulates the expression of multiple isoforms of serum and glucocorticoid regulated kinase1 in mouse cortical collecting duct epithelial cells

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Serum and glucocorticoid regulated kinase1 (SGK1) is a key component of the pathway that leads to activation of the epithelial sodium channel (ENaC) in the aldosterone-sensitive distal nephron (ASDN). Regulation of ENaC activity is a major determinant of renal Na^+ absorption and overall body fluid homeostasis and blood pressure (1, 2). Studies from our laboratory have shown that human skin cells express multiple SGK1 isoforms (a-f) that arise from alternative transcriptional start sites and RNA splicing at the SGK1 locus. The aim of this study was to investigate if SGK1 isoforms are also expressed in the ASDN and to assess their potential role in regulating Na^+ transport. We used the mouse cortical collecting duct cell line mpkCCDcl4 (3) which display aldosterone/sgk1-regulated Na^+ transport (3, 4). Comparison of multiple mouse sgk1 expressed

sequence tags (ESTs) in the mouse EST database (5) with genomic DNA, identified four potential sgk1 isoforms termed sgk1a (original form), 1b, 1c and 1d. Each isoform has a unique amino terminus of varying size but otherwise an identical sequence. Using sequence specific primers, mRNA expression of all four isoforms was confirmed by RT-PCR from purified mpkCCDcl4 cell RNA. Western blotting of protein from mpkCCDcl4 cell lysates overexpressing individually cloned isoforms showed distinct protein bands corresponding to the correct predicted sizes (a~49kDa, b~50kDa, c~48kDa, d~60kDa). Western blot analysis of serum-starved cells exposed to 10 nM aldosterone (Al) or Al plus 1 μM insulin (Ins) showed a time-dependent increase in the endogenous expression levels of multiple sgk1 bands within 1 hr of treatment (n=4). These Al and Al + Ins-induced endogenous sgk1 bands co-migrated with overexpressed sgk1 isoform bands corresponding to sgk1a, 1b, 1c and 1d. However, expression levels of sgk1a and 1b were increased more by Al + Ins treatment compared to sgk1c and 1d. Aldosterone also produced a significant increase in amiloride-sensitive (ENaC-mediated) equivalent short circuit current within 2 hrs of exposure (Con: 6.7 ± 0.1; Al: 12.9 ± 0.8 $\mu A/cm^2$, mean ± sem; $p < 0.001$, one-way ANOVA, n=3), and current reached a peak after 4-6 hrs exposure. Insulin potentiated the Al response at 2 hrs (Al + Ins: 15.8 ± 0.1 $\mu A/cm^2$, n=3; $p < 0.05$) and after 6 hrs (Al: 18.3 ± 1.0; Al + Ins: 21.4 ± 0.6 $\mu A/cm^2$, n=3; $p < 0.05$). Insulin alone had no effect. These data suggest that multiple sgk1 isoforms (1a-1d) are induced by aldosterone which is linked to an increase in ENaC activity. Insulin also potentiated the Al-mediated increase in ENaC activity. Further studies into isoform-specific regulation of Na^+ transport may provide novel mechanisms and possible therapeutic targets for dysregulated Na^+ transport such as observed in primary hypertension.

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PC27

The kinetic and molecular mechanisms of the cadmium-induced mitochondrial swelling

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It is known that Cd^{2+} activates mitochondrial (Mit) swelling following by opening of the permeability transition pore (PTP), fast Ca^{2+} release from the Mit matrix as well as the other pathological processes which may result in the cell damage and death. However the mechanisms contributing to the Mit swelling remain elusive. The aim of this study was to find out the kinetics and molecular mechanisms of the Cd^{2+}-induced Mit swelling.

The experiments were performed on the Mit isolated from adult Wistar rats liver. The rats were anaesthetized by inhalation narcosis with diethyl ether. Mit were obtained by differential centrifugation (1). Mit swelling was measured spectrophotometrically and analyzed by three parameters: i) maximal rate of the process; ii) process lag-time; iii) peak amplitude of the response (2).

We showed that increase of extra-Mit concentration of Cd^{2+} (1-50 μM) caused decrease in the lag-time and the peak amplitude of the Cd^{2+}-induced Mit swelling, but increased the rate of the process. The dependence of the rate of Mit swelling on added Cd^{2+} was sigmoid (EC_{50} = 7,276±1,750 μM, V_{max} = 1,084±0,195 A_{540}/min×mg protein; n = 5). We also found that incubation of Mit with PTP inhibitor – cyclosporin A (10 μM, 1 min) did not prevent the Cd^{2+}-induced Mit swelling. In particular, the rate of the process stimulated by 15 and 50 μM of Cd^{2+} was 61±5 % (n = 6; p = 0,000) and 71±4 % (n = 6; p = 0,000); lag-time – 193±16 % (n = 6; p = 0,000) and 132±8 % (n = 6; p = 0,001); peak amplitude – 102±4 % (n = 6; p = 0,747) and 108±6 % (n = 6; p =0,403) respectively compare to control. Incubation of Mit with the chelator of SH-groups – dithiothreitol (1 mM, 1 min) completely eliminated the Cd^{2+} (15, 50 μM)-induced Mit swelling. In addition we showed that incubation of Mit with modulator of COOH-groups – 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (1 mM, 1 min) slightly potentiated the Cd^{2+}-induced Mit swelling. In particular, the rate of the process stimulated by 15 and 50 μM of Cd^{2+} was 114±10 % (n = 5; p =0,033) and 125±9 % (n = 5; p =0,013); lag-time – 74±9 % (n = 5; p =0,010) and 85±10 % (n = 5; p =0,170); peak amplitude – 108±5% (n = 5; p =0,031) and 105±7 % (n = 5; p =0,356) respectively compare to control.

Thus, our results detailed the main kinetic parameters of the Cd^{2+}-induced Mit swelling in hepatocytes and strongly suggested that this process is not mediated by the opening of PTP, however is significantly regulated by SH- and COOH-groups of the inner Mit membrane.

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PC28

Functional role of a highly conserved sequence motif in the insect amino acid transporter KAAT1

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KAAT1 is an insect amino acid transporter, member of the NSS/SLC6 family of solute carriers, with peculiar functional properties, being activated by Na^+ and by K^+ (1). KAAT1 sequence shows a stretch of three consecutive glycines (Gly85 - Gly87)

that is highly conserved between members of the family (2, 3) and that, according to topology prediction algorithms and to the crystal structure of the NSS/SLC6 member LeuT (4) is located in the extracellular loop 1 (EL1), near the access of the permeation pathway.

In the present study we have investigated the functional role of this region by KAAT1 site-directed mutagenesis and expression in *X. laevis* oocytes. The substitution of each glycine with alanine determined a reduction of transport activity of 40 - 50 % compared with wild-type (wt) (S.E. of 5%, n = 56). The shift of the glycine stretch toward the N or the C terminus of the protein, obtained by the synthesis of the double mutants N84G/G87A and G85A/A88G, reduced the activity respectively to 13% ± 1 (n = 25) and 8% ± 2 (n = 27) of wt, whereas the single mutants N84G and A88G showed a residual transport activity that was respectively 36 % ± 6 (n =31) and 5 % ± 1 (n = 38) of wt.

Our results indicate that EL1 residues influence KAAT1 activity and that in particular, the conserved three-glycine stretch constitutes a "functional motif" in which not the single glycine residue, but the position of the entire motif plays an essential role in transport function.

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PC29

Effect of the brominated flame retardant pentabromophenylether on the function of eel aquaporin 3 expressed in MDCK type II cells

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The European Eel (Anguilla Anguilla) is a euryhaline teleost, which can survive in a wide range of salinities. During adaptation to a saltwater environment there are changes in the expression levels of a number of aquaporin water channels, including eAQP1 and eAQP3 (Lignot et al., 2002; Martinez et al., 2005). Recently we demonstrated that eAQP3 has a limited permeability to H_2O, but causes significant increases in both urea and methylammonium uptake (Walton *et al.*, 2008). Over the last 30 years there have been dramatic falls in worldwide populations of eels. This decrease in eel population coincides with an increase in the levels of environmental brominated flame-retardants (BFRs). In the current study we investigated the effect of the BFR pentabromophenylether (PDBE) on the function of eAQP3.

eAQP3 was subcloned into the eGFP expression plasmid pEGFP-C2. MDCK II cells were grown on permeable filters and transiently transfected with 1μg of cDNA encoding either eGFP-AQP3 or the eGFP control plasmid. To determine the cellular localization of eAQP3 the cells were fixed and viewed using confocal microscopy. Urea uptake experiments were performed on single monolayers. The cells were exposed to an uptake solution (in mM, Mannitol 280, Urea 1, $CaCl_2$ 1.8, $MgCl_2$, 1, KCl 2, HEPES 5, pH7.4 supplemented with 2μCi/ml ^{14}C-Urea) for 90s, followed by several washes in an ice-cold stop solution (in mM Mannitol, 270, Urea 10, $CaCl_2$ 1.8, $MgCl_2$ 1, KCl 2, HEPES 5, pH 7.4). The cells were dissolved in 10% SDS and ^{14}C-urea measured by scintillation counting. All uptake values are quoted as mean ± SEM and the units are pMol urea.cm^2.90s^{-1}. Statistical analysis has been performed using paired or unpaired t-tests as appropriate and significance is assumed at the 5% level. PDBE was prepared as a 1mM stock in DMSO.

When expressed in MDCK cells, eAQP3 was delivered to both the apical and basolateral membranes. In monolayers transiently transfected with eGFP-eAQP3 there was a significant increase in urea uptake compared to monolayers transfected with the control plasmid (eAQP3, 973.0±229.3; control 537.8±223.9 p=0.01, n=3). A 1hr incubation in 1μM PDBE caused a significant reduction in urea uptake in eAQP3 expressing cells (eAQP3, 1276.0 ± 224.3, n=8; eAQP3 + PDBE, 630.8 ± 79.5, n=8, p=0.02) but was without effect on control cells (eGFP, 379.4 ± 48.3, n=8; eGFP + PDBE, 302.1 ± 48.1, n=8, p=0.28). In addition we found that treatment with PDBE caused a redistribution of eGFP-eAQP3 fluorescence from the cell membrane into a cytoplasmic compartment.

In summary we have shown that eGFP-tagging at the N-terminal does not the affect the ability of the eAQP3 to transport urea. In addition exposure to PDBE inhibits eAQP3. This effect of PDBE is most likely due to an internalization of eAQP3.

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PC30

Cell-specific immune and stress signalling in *Drosophila* malpighian tubules confer organismal survival

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The survival of organisms depends on their response to external stressors, such as infection, osmotic or ionic stress, or exposure to xenobiotics or toxins. As barriers between the external and internal environment, epithelia play key roles in defending against osmotic, oxidative and immune stress. Genetic and physiological studies in model organisms, such as Drosophila, have provided clear links between manipulation of stress-associated genes and physiological output, and for many purposes the available genetic tools for Drosophila make it a system of choice. As well as the whole-organism studies that have dominated stress research, powerful genetic tools provide the capability to drill down to specific cells or tissues. The Drosophila

Malpighian tubule, analogous to vertebrate kidney and liver, is an epithelial model for cell-specific, organotypic ion transport and cell signalling studies; and for functional genomics and gene discovery. The tubule is a NO/cyclic cGMP (cGMP)-modulated fluid-transporting epithelium (1) critical for osmoregulatory and detoxifying functions (2,3). Our recent work has suggested that the Drosophila renal (Malpighian) tubule plays a key role in organismal defence against a wide range of stresses, not just osmotic. Tubules are immune tissues (4,5) which express anti-microbial peptides via the IMD (4) and Toll pathways (flyatlas.org).

We show here that a tubule-specific cGMP-kinase (cGK) 'switch' modulates Nf-kB orthologue (Relish) activation, and IMD signalling in tubules. This cGK 'switch', targeted to only principal cells of the tubule, modulates survival of immune-challenged whole flies, providing the first evidence of cyclic nucleotide modulation of innate immunity. We have also shown that tubules are the first-line defence in bacteria-fed Drosophila and so are key 'sentinels' of immune challenge.

Due to the high rates of metabolic activity in epithelia, they can be major sources of ROS in the organism, and thus must be equipped for stress sensing and responses. Tubules have specific adaptations to counter high mitochondrial activity and ROS production including enriched expression of 'antioxidant' genes including superoxide dismutase (SOD), mitochondrial SOD and catalase.

We also show that the tubule is a key stress-sensing tissue for the whole organism, and that manipulation of specific genes in only tubule principal cells is sufficient to modulate organismal stress and immune responses.

Using functional data, and data obtained from modelling gene networks, the integration of stress and immune signalling pathways in the tubule will be presented.

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PC31

Hereditary haemochromatosis protein (HFE)-dependent regulation of iron transfer across placenta

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BACKGROUND AND AIMS: HFE functions as an upstream regulator of liver hepcidin which has been demonstrated to be a negative regulator of intestinal dietary iron absorption and efflux of recycled iron from macrophages. Hepcidin has also been proposed to be a negative regulator of iron efflux from placenta, however it is not known if this of maternal or foetal origin.

Furthermore, HFE has been demonstrated to be present in the placenta, but its role in the dynamics of maternal foetal in iron transfer independent of hepcidin is unknown. In this study we investigated the effects of HFE and dietary iron levels on transfer of iron from mother to foetus in order to determine the importance of maternal and foetal HFE status on iron transfer.

MATERIALS AND METHODS: HFE knockout (KO), wild type (WT), and heterozygote (HET) dams were fed 50 and 200ppm iron diets and mated with HET male mice to produce pups of all genotypes. Dams and pups were sacrificed and pup liver iron levels were determined. mRNA levels of iron transporter genes (Ferroportin1, TfR1, DMT1+IRE, and DMT1-IRE) were determined in placental tissue by real-time PCR.

RESULTS: Liver iron levels of HET pups from KO dams fed 50ppm iron diet were considerably higher (137.42 + 9.19 μg/g dry weight, n = 10) than those from WT dams (112.90 + 3.71 μg/g dryweight, n = 10). This difference was also apparent in the pup liver iron levels when the dams were fed 200ppm iron diet. Iron transporter gene expression was higher in HET pups from KO dams compared to those from WT dams fed 50ppm iron diet but this difference was not significant in HET pups from dams fed 200ppm iron diet.

There was no difference in liver iron levels of KO (115.9 + 7.09 μg/g dryweight, n = 10) and WT pups (113.47 + 3.61 μg/g dry weight, n = 10) from HET dams fed 50ppm iron diet. This was mirrored in the gene expression data, which showed no significant difference between all of the placental genes tested (see above). However liver iron levels of KO and WT pups from HET dams fed 200ppm iron diet were higher in KO pups (146.95 + 9.34 μg/g dryweight, n = 10) than WT (122.08 + 4.74 μg/g dryweight, n = 10). In addition, expression of placental iron transporter genes was also found to be significantly elevated in KO pups than WT.

CONCLUSIONS: Maternal genotype plays a role in regulating placental iron transfer independent of dietary iron content. However, foetal genotype seems to affect liver iron accumulation and the expression of certain iron transporter genes only with adequate iron intake.

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

PC32

Involvement of ryanodine receptors in trophoblast cell-line volume regulation

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The human placenta develops from trophoblasts of the blastocyst and provides an interface of communication and exchange of material between mother and fetus. Trophoblasts are frontline epithelial cells involved in such activities, in which

Ca2+-signalling proteins are richly expressed (Moeau et al., 2003). Intracellular free calcium ions ([Ca2+]i) are a key second messenger, participating in regulation of various cellular activities (Berridge et al., 2003). In the placenta, transcellular Ca2+ transport through trophoblasts is essential in formation of the fetal skeleton (Belkacmi et al., 2005). Ryanodine receptors (RyRs) are high conductance cation channels that mediate Ca2+ release from intracellular Ca2+ to the cytoplasm. To date, the presence and physiological roles of RyRs in trophoblasts have not been reported. A pilot study from our laboratory revealed that functional RyRs are expressed in model trophoblast cell lines. In the current study, RNA encoding RyRs was confirmed in human trophoblast cell-lines (BeWo, JAR, JEG-3 and SGHPL-4) using reverse transcription-PCR. The presence of RyR1 and RyR accessory protein, triadin, were demonstrated in BeWo and JEG-3 trophoblastic cells by immunoblotting and indirect immunofluorescence microscopy. Change of [Ca2+]i was monitored by loading the cells with fura-2 acetoxymethyl ester (AM) and by using fluorescent videomicroscopy system. When BeWo cells were exposed to 1 μM ryanodine, an initial 12.7 ± 1.0% (mean ± standard error in mean (SEM), n = 4) increase of mean fura-2 ratio (i.e. increased [Ca2+]i) was observed. When these cells were treated with 500 μM 4-chloro-meta-cresol (CmC), an RyR1 and RyR2 activator, an initial increase in mean fura-2 ratio of 27.3 ± 1.5% (mean ± SEM, n = 6) was detected, followed by a rapid decrease of signal. Alterations in cell morphology detected by using brightfield videomicroscopy were also observed in these cells in response to RyR activators. Cells were loaded with calcein AM (Crowe et al., 1994) to monitor if changes in cell morphology were due to change in cell volume. Addition of 500 μM CmC caused decrease in calcein fluorescence intensity/initial intensity ratio of 11.8 ± 0.6% (mean ± SEM, n = 4) within 6 minutes in BeWo cells, indicating an increase in cell volume following RyR activation. These findings demonstrate that BeWo and JEG-3 express multiple RyR subtypes, along with certain accessory proteins, and that these channel complexes participate in cell volume regulation.

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PC33

Modified dipeptides affect the growth of the human pancreatic cancer cell line AsPc1

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In addition to being the major route of uptake of dietary nitrogen from the small intestine, expression of the proton-coupled di- and tri-peptide transporter PepT1 is also up-regulated in many cell lines derived from cancerous tissue, despite not being expressed in the wild-type tissue (Mitsuoka et al. 2008). Included amongst these tissues is the pancreas, and PepT1 expression in the human pancreatic cell line AsPc1 has been documented (Gonzalez et al 1998). Here we report that some small modified dipeptides that are PepT1 substrates have an anti-proliferative effect on the growth of AsPc1 cells.

To test the effect on proliferation rates, compounds were included at a concentration of approximately twice their affinity for PepT1 in the medium of AsPc1 cells plated at an initial density of 50% in 96-well plates. After 2-3 days growth under standard conditions (RMPI 1640 medium, plus or minus test compound, supplemented with 10% FCS and penicillin/streptomycin, 37∞C, 5% CO2), the proliferation was measured using a colourmetric assay, either MTT (ATCC, LGC Promochem, UK) or XTT (Cambridge Bioscience, UK). Data in the presence or absence of the compounds (control) was compared by Student's paired t-test between the conditions, and are presented as normalised to the relevant control, mean +/- SEM of n independent experiments.

As can be seen from the data in Figure 1, the compounds DF5:15A and DF5:15B, which are modified simple dipeptides, were able to significantly inhibit the proliferation of AsPc1 cells at 0.1mM (both $p<0.05$, n=4), whilst control dipeptides (Gly-L-Gln at 0.2mM and the hydrolysis-resistant L-Phe-(ψS)-L-Ala at 1.2mM) and the free modified amino acid component of the dipeptide (X at 1mM) did not ($p>0.05$, n=11, 8 and 7, respectively). As a positive control, the anti-cancer agent 5-fluorouracil (5FU, 100 μM) produced a similar level of inhibition of cell proliferation in our assays ($p<0.05$, n=17). The findings above are suggestive of an involvement of PepT1 in the observed anti-proliferative effects on AsPc1 cells in that only the addition to the medium of intact modified dipeptides had an effect, supported by the fact that the concentration of compound needed was similar to the PepT1 affinity measured in rabbit PepT1 (expressed in *Xenopus laevis* oocytes, data not shown). If this is indeed proved to be the case, then compounds like these could be therapeutically useful as anti-proliferative agents against cancerous cells that ectopically express PepT1.

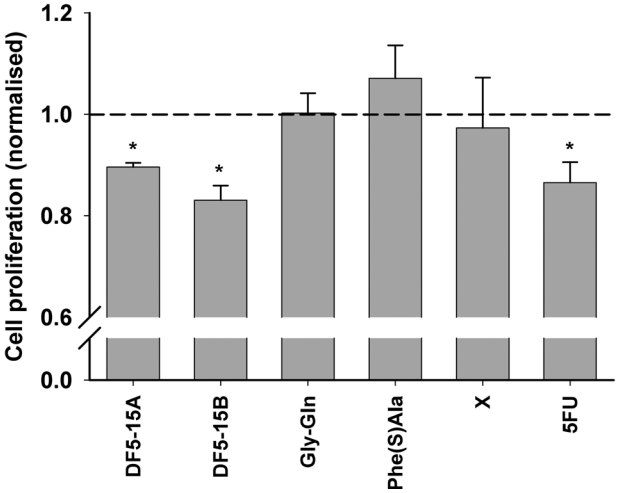

Figure 1: Effect of compounds on proliferation of AsPc1 cells (see text for details) expressed as fraction of cell growth in presence of vehicle only (* $p<0.05$, Student's paired t-test).

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PC34

The role of the different N- and C- termini of the two splice variants of ZnT5 in intracellular localisation

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Two splice variants of the human SLC30A5 Zn transporter gene have been reported in the literature (Cragg et al, 2002, Kambe et al, 2002). The sequences reported differ at their N- and C-terminal regions, corresponding with the use of different 5' and 3' exons (Jackson et al, 2007). The ZnT5 splice variants adopt different subcellular localizations when expressed as fusions to GFP from the corresponding transgenes introduced into Chinese hamster ovary cells. Variant A is expressed in the Golgi apparatus and variant B is expressed throughout the cell, including at the plasma membrane (Jackson et al, 2007). Plasma membrane localization of variant B, has also been observed in human intestinal Caco-2 cells (Cragg et al, 2002), and we have also reported previously localization of ZnT5 to the apical enterocyte membrane in human small intestine, using an antibody that may recognize both splice variants (Cragg et al, 2005), however the differential targeting sequences of the two variants has yet to be defined. Here we report differential localisation of ZnT5 in HeLa cells, when expressed as either an N- or C-terminal GFP fusion.

Plasmid constructs from which one or other of the ZnT5 splice variants have been expressed with either a C-terminal or N-terminal GFP tag were transiently transfected into HeLa cells. Forty eight hours post transfection, cells were visualized by fluorescence microscopy. Dual labelling with markers of specific subcellular compartments was also carried out. Results for Variant A expressed with a C terminal GFP tag confirm localisation at the Golgi apparatus, as previously reported (Kambe at al, 2002), however the same variant expressed as an N-terminal GFP fusion was observed only at the endoplasmic reticulum (ER), confirmed by co-localisation with an ER marker. A potential cleavage site at the N-terminal region of variant A, identified through bioinformatics could explain this pattern of localisation and we hypothesise that the mature variant A peptide localises to the Golgi apparatus and the cleaved GFP N- terminal sequence is visualised at the ER. We will confirm this hypothesis with Western blotting.

In transfected HeLa cells, Variant B is localised to the ER when expressed as either an N-terminal or C-terminal GFP fusion and analysis of the sequence at the N-terminal region does not predict the same cleavage site as observed with variant A, however an ER localisation signal is observed in the C-terminal region. The results from this study are beginning to elucidate the role of the different N- and C- termini of the two splice variants in intracellular localisation/targeting.

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PC35

Diabetes mellitus and gene expression of 'sweet' taste receptors in the jejunum and kidney

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The facilitative glucose transporter, GLUT2, is normally poorly expressed at the jejunal brush-border membrane (BBM), but levels increase markedly in response to the high luminal glucose levels that occur during digestion (Kellett et al., 2000). Recently, the sweet taste receptors T1R2 and TIR3 have been shown to detect high levels of glucose in the jejunal lumen, and to act as signals for GLUT2 insertion at the BBM (Mace et al., 2007). In the kidney, raised levels of glucose are present in the proximal tubular (PT) lumen in diabetic hyperglycaemia, and we have shown that GLUT2 is highly expressed at the PT BBM in streptozotocin (STZ)-diabetic rats and accounts for the increased BBM glucose uptake that occurs in this model (Marks et al., 2003). Unlike the small intestine, expression of taste receptors in the kidney, and their possible involvement in BBM GLUT2 expression, is unknown. The aims of our study were twofold: first to assess whether taste receptors are expressed in kidney cortex; second to determine the effect of type 1 diabetes mellitus (DM) on T1R expression in both jejunum and kidney.

DM was induced in Sprague-Dawley rats by treatment with streptozotocin (STZ, 55mg.kg-1, i.v). Jejunal sections and kidneys were removed from anaesthetised (pentobarbitone sodium, 55mg.kg-1, i.p.) animals 10 and 30 d after STZ, and from control animals. RNA was extracted from jejunal mucosal

scrapes and kidney cortex. T1R1, TIR2 and TIR3 and β-actin transcripts were analysed by real-time PCR. The comparative delta delta Ct method was used to calculate the relative gene expression.
In kidneys from non-diabetic animals all three taste receptors were expressed; however, T1R1 and TIR3 were highly expressed compared with T1R2, a pattern which was also seen in jejunum. 10 d diabetes reduced renal expression of TIR1 and TIR3 by 97% ($p<0.01$) and 55% ($p>0.05$), respectively. In contrast, 30 d DM increased renal T1R1 and TIR3 mRNA levels by 30% and 22% ($p<0.05$ and $p>0.05$ respectively). In jejunum, 10 d diabetes reduced TIR1 and TIR3 mRNA expression by 78% ($p>0.05$) and 65% ($p<0.005$), respectively; whereas 30 d diabetes reduced the expression of T1R1 by 28% ($p>0.05$), but increased that of TIR3 (34%, $p<0.05$). Our data indicate for the first time that gene expression of sweet taste receptors occurs in the kidney. Diabetes caused complex changes in expression of TIR1 and TIR3 in both kidney and jejunum. The relationship between taste receptor expression and diabetes-induced appearance of GLUT2 at the BBM in both tissues is under study.

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PC36

UPSTREAM STIMULATORY FACTORS (USF-1/USF-2) REGULATE HUMAN HEMOJUVELIN GENE EXPRESSION

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Hepcidin is presumed to be the master regulator of systemic iron homeostasis; its gene expression is affected by inflammation, hypoxia, iron levels, the rate of erythropoiesis, and oxidative stress. Hemojuvelin (HJV), a recently identified protein expressed in muscle, heart, and liver, has been shown to be an upstream regulator of hepcidin expression by acting as a bone morphogenetic protein (BMP) co-receptor. Consequently, HJV genetic mutations lead to the severe iron overload disorder juvenile hemochromatosis. There are two HJV isoforms: a membrane-bound and a soluble form, both of which are proposed to regulate hepcidin expression in response to changes in iron levels in vitro and in vivo. However, there is no information on how HJV is regulated. In this study, we identified E-box elements in the human HJV promoter and investigated the role of upstream stimulatory factors (USF) in the regulation of HJV expression through the E-boxes. By co-transfection experiments, we demonstrated that over-expression of USF transactivated the HJV promoter. This was confirmed with electrophoretic mobility shift assays which showed that recombinant USF1 and USF2 bound specifically to the HJV E-box. Our data suggests that USF transcription factors are important regulators of HJV expression, and strengthens the link between USF and iron metabolism.

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PC37

An endocannabinoid regime the functioning of salivary glands

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An endocannabinoid is an important regulatory system for the many physiological functions in all vertebrate organisms. There are a lot of clinical data describing the role of endocannabinoids in the function of salivary gland. However their role in salivation remains almost unclear.
Experiments were performed on male Wistar rats (6–7 weeks old, 100–150 g). Gland was removed after intraperitoneal injection of pentobarbital. Submandibular salivary gland acinar cells were isolated by collagenase treatment. Calcium concentration was estimated by arsenazo III. Protein content was determined using Lowry method.
We showed that *in vitro* application of synthetic agonist of CB1 and CB2 cannabinoid receptors - WIN 55,212-2 (5 μM) to Ca^{2+}-free extracellular solution (1 mM EGTA, 5 min) decreased total calcium content on 39±6 % ($p<0,001$, n=6). At the same time the agonist of the CB2 cannabinoid receptors - virodhamine (30 μM) caused the decrease of calcium content in isolated acini on 28±4 % ($p<0,001$, n=8). After that we found that addition of 2 mM $CaCl_2$ (for 5 min) in the presence of virodhamine evoked significant increase of total Ca^{2+} content in the acinar cells on 130±22 % ($p<0,001$, n=6) whereas in control - on 57±15 % ($p<0,01$, n=6).
In addition, when WIN 55,212-2 (5 μM) and virodhamine (30 μM) was applied to isolated acini separately in the presence of thapsigargin (0,1 μM) in Ca^{2+}-free medium (1 mM EGTA, 10 min) we observed a decrease in the total calcium content on 30±6 % ($p<0,01$, n=8) and 19±3 % ($p<0,001$, n=8) accordingly to the effect of thapsigargin only. The latter suggest the potentiation of the Ca^{2+} release from the endoplasmic reticulum upon activation of CB1 and CB2 receptors. We have also found that addition of 2 mM $CaCl_2$ (for 5 min) in the presence of the mixture of WIN 55,212-2 and thapsigargin occurs dramatic increase of the total Ca^{2+}-content on 310±34 % ($p<0,01$, n=6) whereas in the presence of thapsigargin only - on 126±41 % ($p<0,05$, n=9). Likewise, the addition of 2 mM $CaCl_2$ (for 5 min) in the presence of the mixture of virodhamine and thapsigargin induces increase of the total Ca^{2+}-content on 213±46 % ($p<0,001$, n=7) whereas the thapsigargin only - on 108±38 % ($p<0,001$, n=7).
In conclusion, our data suggest that endogenous CB1 and CB2 cannabinoid receptors contribute to the regulation of submandibular salivary gland and this regulation occurs via Ca^{2+}-dependent mechanisms (Ca^{2+} release from the endoplasmic reticulum and consequent store-operated Ca^{2+}-entry).

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