C01 and PC01

Protective role of calmodulin in alcohol-induced trypsinogen activation

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Acute pancreatitis is generally initiated by premature trypsinogen activation in pancreatic acinar cells mediated by excessive intracellular calcium release from internal stores. One of the major causes of acute pancreatitis is excessive alcohol intake, but the molecular mechanism of this severe inflammatory disease is not completely understood. We now show that in two-photon permeabilized mouse pancreatic acinar cells even a relatively low ethanol concentration as well as its non-oxidative metabolite palmitoleic acid ethyl ester (POAEE) elicit calcium release from intracellular stores and also induce intracellular trypsinogen activation (1, 2). Reintroducing the calcium sensor calmodulin (at a normal intracellular concentration found in intact cells) to the permeabilized cells dramatically reduced ethanol-induced calcium release and trypsinogen activation. Pre-incubation of cells with a calmodulin activator CALP-3 (100 mkM) abolished the ethanol and POAEE effects in intact and permeabilized cells (1). Both ethanol-elicited and POAEE-induced calcium liberation and trypsin activity were significantly reduced in acinar cells from mice in which type 2 inositol trisphosphate receptors had been knocked out. Double knock out of inositol trisphosphate receptors of both types 2 and 3 further reduced ethanol-induced or POAEE-induced calcium release and trypsinogen activation to very low levels (1, 2). Thus, the calmodulin-sensitive inositol trisphosphate receptor calcium release channels, that are responsible for normal pancreatic stimulus-secretion coupling, also play a major role in the toxic action of ethanol. Calmodulin provides a protective mechanism, regulating the sensitivity of the calcium release process (1). The marked inhibition of ethanol-induced (or POAEE-induced) Ca2+ release and trypsin activation by a calmodulin activator suggests potential therapeutic benefits for treatment of pancreatitis.

Gerasimenko J.V et al (2011) Calmodulin protects against alcohol-induced pancreatic trypsinogen activation elicited via Ca2+ release through IP3 receptors. PNAS, 108(14):5873-8

Gerasimenko J.V. et al (2009) Pancreatic protease activation by alcohol metabolite depends on Ca2+ release via acid store IP3 receptors. PNAS, 106(26):10758-63.

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

C02 and PC02

Calcium regulation of apoptosis in pancreatic acinar cells

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We have studied calcium regulation of induction of apoptosis in pancreas. In pancreatic acinar cells, the earliest events were found to be cytosolic calcium elevations due to release of calcium from intracellular stores1. As a result of that calcium levels also increased in mitochondria aiding mitochondrial depolarisation and mPTP. High mitochondrial calcium at the time of oxidant stress was found to be the crucial factor in the cell fate2. When mitochondrial calcium was low, then apoptosis did not occur regardless of other stores' content. We also studied Bcl-2 family members, well known regulators of apoptosis involved in regulation of intracellular calcium homeostasis3. Most interesting was a potential link between Bcl-2 family proteins and a calcium induced calcium release (CICR) from the intracellular stores. Inhibition of antiapoptotic proteins induced calcium release from the ER that lead to the formation of calcium plateau while inhibition of either IP3Rs or RyRs reduced but did not abolish calcium release. Furthermore, we have shown that loss of Bcl-2 protein significantly affected calcium extrusion as well as apoptosis/necrosis ratio in pancreatic acinar cells. In conclusion we suggest that Bcl-2 regulates vast majority of main components of calcium signalling, serving its crucial role in regulation of cell death.

Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV. (2009) Journal of Biological Chemistry 284(31): 20796-803

Gerasimenko O, Gerasimenko J. (2010) Methods in Molecular Biology 591: 201-10.

Gerasimenko J, Ferdek P, Fischer L, Gukovskaya AS, Pandol SJ. (2010) Pflügers Archiv 460(5): 891-900.

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

C03 and PC03

Indentification and characterisation of ZnT10

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Zn is an important element throughout the body, essential for many proteins and enzymes. Homeostasis of Zn is undertaken by Zn transporters of which there are

two families; ZIP and ZnT (Lichten and Cousins, 2009). Little is known about the role and expression pattern of ZnT10, however in silco data predict expression to be restricted to foetal tissue (Seve et al., 2004). We have shown by analysis of a human tissue panel by qPCR a restricted expression profile for ZnT10 in adult human tissue. Expression was identified in the cervix, liver, brain, ovary, testes, small intestine and colon at the mRNA level. Bioinformatic analysis of ZnT10 has confirmed ZnT family topology, with 6 conserved transmembrane spanning domains (Seve et al., 2004, TMPred, TMHMM) and a CDF motif predicted to lie between amino acids 34-50 (ScanProsite). In addition, a cleavage site between amino acid position 29 and 30 is predicted (SignalP). Three N-, one O-glycosylation and fifteen possible phosphorylation sites have been predicted (NetNGlyc, NetOGlyc). Using qPCR we have identified a decrease at the mRNA level of ZnT10 in response to extracellular Zn2+ in Caco-2 cells (0.37 ± 0.05 compared with 9.7 x 10-2 ± 0.03 at 150 μM Zn2+ (n = 3), when expressed as a ratio of ZnT10:GAPDH, $p<0.01$ by Student's t test). This response for a ZnT family member is not without precedence; ZnT5 also shows down regulation in response to extracellular Zn. A putative Zn responsive element (ZRE) has been identified in the promoter region of ZnT5 (Coneyworth and Ford, Unpublished data). Deletion of this sequence and mutations of specific base pairs in this element eliminated the response to Zn. We dissected the 5'-UTR of ZnT10 for a putative ZRE consensus sequence. A 3 kb upstream region from the ATG was screened using the nucleotide alignment package, Fuzznuc. This identified a putative ZRE 72 bp upstream of the translational ATG start site. To measure activity, a reporter construct comprising the E-Coli β-galactosidase gene downstream of a 942 bp product containing the putative ZRE was generated. In Caco-2 cells transiently expressing this construct, β-galactosidase activity decreased significantly in media containing 150 μM extracellular Zn2+ when compared with untreated cells expressing the same construct, indicating decreased transcription from the putative promoter (Normalised mean β-galactosidase activity (adjusted by control for each experiment) (U/mg) 1.00 ± 0.05 compared with 0.74 ± 0.07 at 150 μM Zn2+ (n = 14), $p < 0.006$ by Student's t-test). The ZRE in this construct has been mutated and will be analysed in this system. In summary we have identified that ZnT10 is differentially expressed in human adult tissues is down regulated at the mRNA level in response to Zn, potentially mediated through transcriptional regulation involving a ZRE in the putative promoter region of ZnT10.

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Seve, M., F. Chimienti, et al. (2004). "In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression." BMC Genomics 5(1): 32.

This work was supported by a MRC studentship (to H.J.B)

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

C04 and PC04

Mirror-like SeSAME/EAST renal phenotype in mice lacking the pH-sensitive Kir5.1 (Kcnj16) K^+ channel subunit

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The heterotetrameric association of Kir4.1 and Kir5.1 subunits forms a 45 pS, inwardly-rectifying and pH-sensitive, K+ channel which underlies the major component of the basolateral membrane K+ conductance along the distal nephron (1,2,3). The functional importance of Kir4.1/Kir5.1 channel in renal ion transport has recently been highlighted by the identification of mutations in the human Kir4.1 gene which result in altered function of Kir4.1/Kir5.1 channel, a reduction in ion transport by the distal convoluted tubule (DCT) and ultimately in SeSAME/EAST syndrome, a complex disorder that includes salt wasting and hypokalaemic alkalosis (4,5).

The extreme sensitivity of the Kir4.1/Kir5.1 channel to intracellular pH (pHi) is confered by the Kir5.1 subunit. Here, the role of the Kir5.1 subunit in renal function was investigated using mice with a targeted disruption of the Kir5.1 gene (Kcnj16).

Mice were maintained in metabolic cages and urine was collected daily by non-invasive methods. Venous blood was taken from the retroorbital plexus of conscious animals. The Kir5.1-/- mice displayed hypercalciuria with hypokalaemic hyperchloremic metabolic acidosis (mean ± SE) (Kir5.1-/- : pHplasma = 7.19 ± 0.015, n = 10; Kir5.1+/+ : pHplasma = 7.35 ± 0.015, n = 11 ; $P < 0.0001$, Student t test). Addition of furosemide, an inhibitor of apical Na+/K+/2Cl- cotransporter in the thick ascending limb, to the powdered chow, or subcutaneous injection of amiloride, an inhibitor of apical Na+ channel (ENaC) in the aldosterone-sensitive distal nephron, revealed no significant alteration in ion transport by these structures in Kcnj16 deficient mice. By contrast, the increase in Na+ urine excretion observed in Kir5.1-/- mice (8.2 ± 3.8-fold, n = 12) in response to a short-term exposure to hydrochlorothiazide (HCTZ, i.p. injection), an inhibitor of ion transport in the DCT, was significantly higher than that observed in Kir5.1+/+ mice (2.9 ± 0.4-fold, n = 10; $P < 0.05$, Student t test), indicating an excessive salt absorption in DCT cells of Kir5.1-/- mice. Furthermore, a 4-day treatment with HCTZ added to the powdered chow normalized urinary excretion of sodium and calcium, and abolished acidosis in Kir5.1-/- mice.

Single-channel patch clamp recording of K+ channels in the DCT basolateral membrane of Kir5.1+/+ and Kir5.1-/- mice revealed that Kcnj16 deletion led to an increased membrane K+ conductance (Kir5.1-/- : Po^*g = 7.94 ± 0.62 pS, n = 10; Kir5.1+/+ : Po^*g = 5.12 ± 1.12 pS, n = 7 ; $P < 0.05$, Student t test), due to the high and pH-insensitive open probability of the remaining homomeric Kir4.1 channels in Kir5.1-/- mice.

In conclusion, disruption of Kcnj16 in mice induces an increase in ion transport activity in DCT cells, leading to a severe renal phenotype which, apart from hypokalaemia, is the opposite of the phenotype seen in SeSAME/EAST syndrome and highlights the important role played by Kir5.1 as a pH-sensitive regulator of renal function.

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Bockenhauer D et al. (2009) N Engl J Med 360:1960-1970.

Scholl UI et al. (2009) Proc Natl Acad Sci U S A 106:5842-5847

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

C05 and PC05

Non-genomic Effects of Oestrogen on Airway Surface Liquid Dynamics and Ion Transport of Normal and Cystic Fibrosis Bronchial Epithelia

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Male cystic fibrosis (CF) patients survive 9 years longer than females ("CF gender gap") and lung exacerbations in CF females vary during the estrous cycle. Estrogen has been reported to reduce the height of the airway surface liquid (ASL) in female CF bronchial epithelium. Here we investigated the effect of estrogen on ASL height and ion transport in normal (NuLi-1) and CF (CuFi-1) bronchial epithelium monolayers grown on permeable filters in an air-liquid interface. Confocal fluorescence microscopy experiments revealed that ASL height was significantly higher in the non-CF cell line compared to the CF cells (NuLi 6.82 ± 0.33μm vs CuFi 5.58 ± 0.14μm, n=20, p<0.001). 17β-estradiol (E2, 0.1 to 10nM) reduced the ASL height in both non-CF (25% decrease, n=5, p<0.05, ANOVA) and CF (20% decrease, n=5, p<0.05, ANOVA) cell lines after 30 min treatment. Treatment with the Cl- transport inhibitor bumetanide (10μM) or the KCNQ1 channel blocker chromanol HMR1556 (1μM) decreased ASL height significantly in both cell lines. However, E2 had no additive effect on ASL height in the presence of these ion transporter inhibitors. Moreover E2 decreased the bumetanide-sensitive Cl- current in normal cells (E2: 6.47 ± 2.08 μA/cm2, Control: 9.52 ± 2.08 μA/cm2, n=3, p<0.05, paired t-test) and produced an increase in amiloride (10μM) sensitive current in CF cells (E2: 11.097 ± 1.805 μA/cm2, Control: 8.801 ± 1.464 μA/cm2, n=7, p<0.05, paired t-test). Treatment with the nuclear-impeded Estrogen Dendrimer Conjugate (EDC 0.1 - 1nM E2 equivalent concentration) produced a significant reduction in ASL height in CF and non-CF cells (4.72 ± 0.25μm in NuLi-1 and 4.86 ± 0.42μm in CuFi-1, n=5, p< 0.05, ANOVA)

whereas the empty dendrimer had no effect. These results demonstrate that E2 dehydrates both CF and normal ASL and these rapid responses to E2 are membrane-initiated rather than via the classical nuclear receptor signal transduction pathway. The ion transporter inhibitor data indicate that E2 acts on ASL by inhibiting Cl- secretion in normal cells and increasing Na+ absorption in CF cells.

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C06 and PC06

BLINaC: An epithelial Na^+ channel in the liver

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The Brain Liver Intestine Na^+ Channel (BLINaC) is an ion channel of the DEG/ENaC gene family. Its physiological function is completely unknown. Expression of rat BLINaC (rBLINaC) in Xenopus oocytes leads to small unselective currents that are only weakly sensitive to amiloride[1]. rBLINaC is potently inhibited by micromolar concentrations of extracellular Ca^{2+} and removal of Ca^{2+} leads to robust currents and increases Na^+ selectivity of the ion pore[3]. Strikingly, the species ortholog from mouse (mBLINaC) has an almost 250-fold lower Ca^{2+} affinity than rBLINaC, rendering mBLINaC constitutively active at physiological Ca^{2+} concentrations[3]. In addition, mBLINaC is more selective for Na^+ and has a 700-fold higher amiloride affinity than rBLINaC[3]. A single amino acid in the extracellular domain determines these profound species differences[3]. These results suggest that rBLINaC is opened by an unknown ligand whereas mBLINaC is a constitutively open epithelial Na^+ channel.

In rodents, the blinac mRNA is expressed mainly in brain, liver, and intestine and to a lesser extent in kidney and lung[1]; in humans, it is mainly expressed in the small intestine[2]. To explore the expression of the BLINaC protein in these tissues, we generated a polyclonal antibody against BLINaC. This antibody revealed prominent expression of BLINaC in the apical membrane of cholangiocytes lining the bile duct of mice, potentially implicating BLINaC in the generation of secondary bile.
Guided by the localisation of BLINaC in cholangiocytes we tested whether the channel is affected by bile. Interestingly the application of diluted bile to Xenopus oocytes expressing rBLINaC induces a strong, amiloride-sensitive Na^+-selective current. This finding suggests the presence of an endogenous activator of rBLINaC in bile and supports the longstanding hypothesis that BLINaC is a ligand-gated ion channel. We are currently trying to unravel the nature of the ligand from bile.
In addition we identified the fenamate flufenamic acid (FFA) and related compounds as agonists of rBLINaC. Application of millimolar concentrations of FFA to rBLINaC

expressing oocytes induces a robust, Na^+-selective current, which is partially blocked by amiloride. We also discovered that rBLINaC and mBLINaC, similar to the related acid sensing ion channels (ASICs), are inhibited by micromolar concentrations of diarylamidines and nafamostat. Thus, we identified pharmacological tools that will help to characterize the function of BLINaC in native cells, such as cholangiocytes.

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[2] Schaefer, L et al. (2000) FEBS Lett. 471, 205–210

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

C07 and PC07

Relationship between thirst perception and plasma arginine vasopressin concentration in man

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It is well established that plasma osmolality is a major determinant of the release of arginine vasopressin (Thompson et. al., 1991) and that oral fluid loads and dehydration show a consistent thirst perception variation in man (Obika et al., 2009). We examined the possibility that measurements of thirst perception using the Visual Analogue Scale (VAS) can be used to estimate plasma arginine vasopressin concentration in man. In thirty normal subjects (males = 15 and females = 15, after giving their informed consent) thirst perception (TP, cm) was rated (Thompson et al., 1991) and 5.0 ml blood samples were collected from the ante-cubital vein for measurement of plasma arginine vasopressin (Pavp, pg/ml) concentration using Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, USA). The table below shows the anthropometric data of the subjects. Although male subjects were statistically significantly older and taller than the females, the body mass indexes (BMI) were similar (24.5 ± 0.5 vs 25.5 ± 0.8 Kg/m2; male vs female), as well as the blood pressures and body weights. There was no statistically significant difference, male vs. female in TP (5.26 ± 0.51 vs 5.39 ± 0.53 cm), calculated plasma osmolality from TP values, i.e., Posm = 10TP/3 + 281 (298.5 ± 1.7 vs. 299.0 ± 1.8 mOsm/kg H20), and measured plasma arginine vasopressin (4.85 ± 0.30 vs. 4.71 ± 0.31 pg/ml). Furthermore, the calculated Pavp from TP, i.e., Pavp = (TP – 1.2)/0.75 (Igbokwe and Obika, 2008) were also similar (5.40 ± 0.69 vs. 5.60 ± 0.70 pg/ml). When Pavp was calculated from plasma osmolality, Pavp = 0.43(Posm – 284.3) (Thompson et al., 1986), the values were also similar (6.10 ± 0.70 vs.6.30 ± 0.80 pg/ml). Although the measured plasma arginine vasopressin concentration was significantly lower than the estimated values from TP and osmolality, there was no statistically significant difference between the Pavp calculated from TP and that from plasma osmo-

lality. It is reasonable to conclude that plasma arginine vasopressin concentration may be estimated using thirst perception rating or plasma osmolality values.

Anthropometric data of subjects. Values are Means ± SEM

	MALE (n = 15)	FEMALE (n = 15)
Age, yr.	27.4 ± 0.62	24.7 ± 0.69*
Weight,Kg.	72.6 ± 1.75	68.7 ± 2.43
Height,m.	1.72 ± 0.01	1.64 ± 0.01*
Body Mass Index, Kg/m2.	24.4 ± 0.5	35.5 ± 0.8
Systolic Blood Pressure, mmHg.	119 ± 1.73	115 ± 1.95
Diastolic Blood Pressure, mmHg.	78 ± 1.25	74 ± 1.89

*p< 0.05.

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

C08 and PC08

Activation of the epithelial sodium channel (ENaC) by the cysteine protease cathepsin S

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There is good evidence that proteolytic processing of the epithelial sodium channel (ENaC) by serine proteases (e.g. furin, trypsin, chymotrypsin, plasmin, elastase, prostasin) is critically important for channel activation [1; 2]. An inappropriate ENaC activation by proteases may be involved in sodium retention and the pathogenesis of arterial hypertension in the context of renal disease [3]. However, at present the molecular mechanisms by which proteases activate ENaC are not yet fully understood. In addition to serine proteases other types of proteases, e.g. cathepsin proteases of the group of cysteine proteases, may be able to activate ENaC under physiological and/or pathophysiological conditions. In particular cathepsin proteases are known to play an important role in inflammatory diseases. As far as we know there are no data yet on the effect of cathepsins on ENaC activity. For our investigation cathepsin S (Cat S) was chosen as candidate protease because Cat S is

expressed in the kidney and plays a role in renal development. Thus, the aim of this study was to investigate the effects of Cat S on human ENaC.
Using the two-electrode voltage-clamp technique we could demonstrate that Cat S activates amiloride-sensitive ENaC whole-cell currents (ΔI_{ami}) in $\alpha\beta\gamma$-human ENaC expressing oocytes. Cat S (1 μM) stimulated ENaC-mediated currents by about two-fold compared to a five- to sixfold stimulatory effect of chymotrypsin applied in a concentration (2 μg/ml) known to elicit a near maximal effect on ENaC. Moreover, we examined the effect of morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl (LHVS), an irreversible and specific Cat S inhibitor [4], on proteolytic ENaC activation. For this purpose, Cat S (1 μM) was pre-incubated with the inhibitor LHVS (2 μM) for ten minutes and then its effect on ENaC currents was tested. The inhibition of Cat S by administration of LHVS abolished the stimulatory effect of Cat S on ΔI_{ami}. In contrast, LHVS (2 μM) had no effect on the activation of ENaC by the serine proteases trypsin and chymotrypsin (different concentrations: 0.02; 0.2; 2 μg/ml).
In summary, our findings reveal for the first time an activation of ENaC by a cysteine protease of the family of cathepsin proteases (Cat S). Furthermore, by using the selective Cat S inhibitor LHVS we showed that Cat S activity is required for the stimulatory effect on ENaC. Our findings indicate that Cat S may play a (patho-)physiological role in proteolytic ENaC activation.

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Passero CJ *et al.* (2010). *Curr Opin Nephrol Hypertens* **19**, 13-19.

Riese RJ *et al.* (1998). *J Clin Invest* **101**, 2351-63.

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C09 and PC09

In vivo **evidence for protease-induced activation of the epithelial sodium channel ENaC in rat collecting duct**

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In recent years, considerable evidence has amassed for the idea that proteases, by cleaving the α or γ subunit of ENaC, can increase the activity of this channel (Hamm *et al*, 2010). This evidence has come from *in vitro* studies, largely using electro-

physiological techniques to assess ENaC activity in heterologous expression systems or in isolated nephron segments (Kleyman *et al*, 2009; Nesterov *et al*, 2008). The present study has assessed the physiological relevance of these observations by measuring sodium reabsorption in rat collecting ducts *in vivo*.
Rats (n = 14) on a standard sodium intake were anaesthetised with thiobutabarbital sodium (Sigma; 110 mg kg^{-1}, I.P.) and prepared surgically for micropuncture. Late distal tubules of superficial nephrons were perfused twice (5 nl min^{-1} for 3-6 min) with a solution similar to native tubular fluid but containing ^{14}C-inulin and ^{22}Na. The first perfusion was always a control solution; the second perfusion was either a control solution (time controls) or a solution containing chymotrypsin at a concentration of 2 μg ml^{-1}. Urinary recoveries of ^{14}C-inulin and ^{22}Na were monitored; if the inulin recovery was >85% of the amount perfused, the urinary ^{22}Na/^{14}C-inulin (Na/In) ratio was calculated.
In time controls, the Na/In ratio did not change significantly (34.3 ± 3.0% vs. 37.0 ± 2.9%; n = 18 pairs, NS, paired t test). In contrast, chymotrypsin reduced the ratio from 34.1 ± 4.0% to 25.6 ± 2.9% (n = 14 pairs, P<0.05, paired t test). These data are consistent with the proposal that proteases, acting from within the lumen, can stimulate ENaC activity in the mammalian collecting duct.

Hamm LL *et al.*, Curr Opin Nephrol Hypertens 2010; 19: 98-105.

Kleyman TR *et al.*, J Biol Chem 2009; 284: 20447-20451.

Nesterov *et al.*, Am J Physiol Renal Physiol 2008; 295: F1052-F1062.

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

C10 and PC10

$P2X_4$ receptors and the epithelial sodium channel in mouse collecting ducts *in vivo*

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Extracellular nucleotides, acting via P2 (purine) receptors, can modify epithelial sodium channel (ENaC)-mediated sodium reabsorption in collecting ducts (Bailey & Shirley 2009), but the P2 receptor subtype(s) involved have not been clearly defined. Pharmacological profiling and the use of genetically engineered mice point to an important role for $P2Y_2$ receptors (Pochynyuk *et al* 2010), but a patch-clamp investigation of rat cortical collecting duct principal cells has provided evidence that apical $P2X_4$ and/or $P2X_{4/6}$ receptors may also modulate ENaC activity (Wildman *et al* 2008). The present study was designed to assess, *in vivo*, the possible role of the $P2X_4$ receptor subunit in the regulation of ENaC activity. $P2X_4$ null mice ($P2X_4^-$

$^{/-}$), and their wild-type littermate controls ($P2X_4^{+/+}$), were anaesthetised (thiobutabarbital sodium, 100 mg/kg, I.P.; Sigma) and surgically prepared for renal clearance studies. Baseline clearance measurements were made for 40 minutes; mice were then given the ENaC inhibitor benzamil (1 mg/kg bolus, IV), and, after a 20 minute equilibration period, clearance collections were repeated for a further 40 minutes. Data are presented as means ± SEM; statistical comparisons were made using ANOVA. In animals used as drug-vehicle controls, glomerular filtration rate (GFR) and fractional sodium excretion (FE_{Na}) remained stable. Benzamil treatment did not affect GFR significantly in either $P2X_4^{+/+}$ or $P2X_4^{-/-}$ mice. In $P2X_4^{+/+}$ mice, benzamil caused an increase in FE_{Na} from 0.6 ± 0.2 % to 1.9 ± 0.3% (n = 8, P<0.001; ΔFE_{Na} = 1.3 ± 0.2 %). In $P2X_4^{-/-}$ mice, benzamil increased FE_{Na} from 0.8 ± 0.2 % to 1.8 ± 0.2 % (n = 8, P<0.001; ΔFE_{Na} = 1.0 ± 0.3 %). The benzamil-induced increase in FE_{Na} was not significantly different between the two groups. These data provide no solid evidence for a role for $P2X_4$ receptor subunits in the regulation of ENaC-mediated sodium reabsorption in mice on a normal sodium intake. Experiments are now under way to assess whether this remains the case when ENaC expression and activity is up regulated under sodium-restricted conditions.

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Pochynyuk O et al. FASEB J 2010; 24: 2056-2065.

Wildman SSP et al. J Am Soc Nephrol 2008; 19: 731-742.

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

C11 and PC11

Disruption of ClC-5 is not associated with defective proton secretion by the collecting duct

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Mutations in CLCN5 gene cause Dent's disease, an X-linked disorder characterized by proximal tubule dysfunction including low-molecular-weight proteinuria, hyperphosphaturia, and hypercalciuria. CLCN5 codes for ClC-5, is a Cl^-/H^+ antiporter expressed in the proximal tubule, the thick ascending limb, and the α-type intercalated cells (αIC) of the collecting duct (CD). At the subcellular level, ClC-5 has been localized to apical endosomes together with the vacuolar H^+-ATPase. Patients harbouring mutations of ClC-5 may show defective urinary acidification and modifications in the polarity and/or expression of vacuolar H^+-ATPase in the proximal tubule and in αIC. Here we investigated the role of ClC-5 in the function of αIC using Clcn5 knock-out KO) mice which are a faithful model of Dent's disease. We chal-

lenged these mice with a chronic NH_4Cl load to establish their physiological response and studied H^+-ATPase activity and trafficking in isolated CDs. Metabolic studies under baseline condition confirmed the presence of hypercalciuria and higher titratable acid excretion which are features of Dent's disease, without any difference in blood parameters. Clcn5 KO mice also exhibited more acidic urine than control (WT) littermates. Mice were anesthetized by a mixture (s.c. 0.1ml/g body weight) of ketamine (10%) and xylazine (5%) and the right carotid artery catheterized. Fresh arterial blood was collected from conscious, mildly constricted mice 3 h after anesthesia. Venous blood samples were obtained from the inferior vena cava immediately after killing. For acid-loading experiments, a solution of 300 mM NH_4Cl (in 2% sucrose) was given to mice in drinking water for 2 or 6 days. Urine was collected daily (16h) under mineral oil before acid-loading experiments (day 0) and after 2 or 6 days of ingestion of NH_4Cl. The body weight, fluid intake and urine flow rate were monitored. After a chronic NH_4Cl load, both Clcn5 WT and KO mice were able to adequately increase their ammoniuria. As expected, WT mice increased the excretion of titratable acid, whereas KO mice did not increase titratable acid above their baseline level. Clcn5 KO mice showed a lower blood pH and bicarbonate compared to WT mice. They also exhibited hyperchloremia and hyperkalemia. We then assessed apical NH_3 uptake and proton secretion by the cortical and outer medullary CDs isolated from chronic NH_4Cl loaded mice (300 mM NH_4Cl 2% sucrose solution as beverage, 6 days before the experiment). Mice were anesthetized with xylazin/ketamin i.p (as above). The CDs were microperfused in vitro (kidneys were cooled in situ for 1 min and then removed) and apical NH_3 uptake and H^+ secretion were measured after cell acidification via an apical NH_4Cl prepulse. Both apical NH_3 entry and H^+ excretion were drastically increased in CDs from Clcn5 KO mice. Furthermore, immunostaining revealed that the α4 subunit of the vacuolar H^+-ATPase was more present at the luminal plasma membrane in outer medulla αICs from Clcn5 KO mice compared to WT controls. For immunostaining, mice were anesthetized with ketamine/xylazin (as above) and perfused through the left ventricle with PBS followed by fixative.

These data reveal that the lack of ClC-5 in mouse is not reflected by defective acid secretion in the CD. On the contrary, mice lacking ClC-5 show a major increase in acid excretion, mediated by increased trafficking of vacuolar H^+ATPase to the apical plasma membrane of αIC, probably to compensate for the generalized proximal tubule defect.

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

C12 and PC12

Loss of neuronal cGMP kinase I abolishes acid-induced HCO_3^- secretion and predisposes mice to bleeding duodenal ulcers

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Background and Aims: Brief contact of the duodenal mucosa with luminal acid elicits a long lasting HCO3- secretory response, which is thought to be one of the major protective mechanisms of the duodenal mucosa against acid damage. The H+-induced signaling pathways are not fully understood, and direct experimental evidence for a direct link between disturbed H+-induced HCO3- secretory response and duodenal ulcer development is lacking. Here we describe the spontaneous development of bleeding ulcers and early death in cGMP kinase type I knockout (cGKI-/-) mice that is amenable to proton pump inhibitor treatment, and investigate the underlying signaling defect. Methods and Results: Anesthetized, luminally perfused cGKI-/- mice were unable to respond to a physiological H+ stimulus with a HCO3- secretory response. Smooth-muscle selective cGKI knockin rescued the motility disturbance but not the defective HCO3- secretion or the ulcus phenotype. H+-induced HCO3- secretion was not attenuated by selective inactivation of the cGKI gene in interstitial cells of Cajal or in enterocytes, but was abolished by inactivation of cGKI in nestin-expressing cells, demonstraing that cGKI in the central nervous system is required for sensing luminal acid and responding to it with increased duodenal bicarbonate output. cGKI was found to be strongly expressed in the brainstem nucleus tractus solitarius. Accordingly, truncation of the subdiaphragmal N. vagus (under isoflurane inhalation anaesthesia: 1.2-2.2% in 30% O_2-70% N_2) significantly diminished H+-induced HCO3- secretion in WT mice, whereas stimulation of the subdiaphragmal N. vagus elicited a similar HCO3- secretory response in cGKI-/-,nestin-cGKI-/- and WT mice. Gastric acidity did not differ between cGKI-/- and WT mice. Conclusions: These findings show that protection of the duodenum from acid injury requires neuronal cGKI, and that its lack results in the formation of duodenal ulcers.

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C13 and PC13

Human erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are up-regulated by *Plasmodium falciparum*

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Plasmodium falciparum relies on anion channel activity in the erythrocyte membrane to ensure the transport of nutrients and waste products associated with its development after invasion. The molecular identity of these "New Permeability Pathways (NPP)" described almost thirty years ago (1), is unknown, but their currents correspond to up-regulation of endogenous channels displaying complex gating and kinetics (2-3). In a previous work (4), we had shown that a maxi-anion channel was present in the human red cell membrane. We now demonstrate that components of the Peripheral-type Benzodiazepine Receptor (PBR) are present and functional in the human erythrocyte membrane, and suggest their implication in the increased permeability of infected cells.

The PBR, originally described in mitochondrial membranes, consists of at least three components: Voltage-Dependant Anion Channel (VDAC), Translocator protein (TSPO), and Adenine Nucleotide Transporter (ANT). First, using RT-PCR, we showed that transcripts for the three subunits can be found in the erythroid cell line and CD34+ cells, compared to Ramos cells as a standard. We also demonstrated by western blots, mass spectrometry and immunofluorescence staining that the proteins are present in the mature human erythrocyte membrane (venous blood obtained from healthy volunteers, with written informed consent; all experiments done at least three times).

Secondly we tested the hypothesis whereas this PBR could be the molecular support of the NPP activity described in P. falciparum-infected erythrocytes. PBR are characterised by nanomolar affinity for the ligands PK11195⟩Ro5-4864⟩Diazepam (5). The three ligands were shown to be more potent in inhibiting *P. falciparum* growth *in vitro* than NPPB, a classical NPP inhibitor. IC_{50} were 13.2, 62.8 and 80.5 μM for PK11195, Ro5-4864 and diazepam, respectively, compared to 97.6μM for NPPB (n=3). These ligands also reduced membrane transport of infected erythrocytes, as seen by isosmotic sorbitol haemolysis (characteristic of NPP activity). Analysis showed that the half-times of lysis ($t_{1/2}$) was significantly increased ($P\langle 0.001$, student's t-test) when concentration reached 50 μM, 1 μM and 10μM for PK11195, Ro5-4864 and Diazepam, respectively (n=3). Using the whole-cell configuration of the patch clamp technique, we also showed (n=6 for each condition) that the three ligands reduced the conductance in P. falciparum-infected erythrocytes (representative traces are shown in figure 1).

These data support the hypothesis that the dormant PBR mediates the band 3-independent anion conductance in normal erythrocytes, and, after up-regulation by *P. falciparum*, becomes the "New Permeability Pathways" in infected erythrocytes. These channels are obvious targets in the fight against malaria.
All these results are included in an article currently in press in Blood journal.

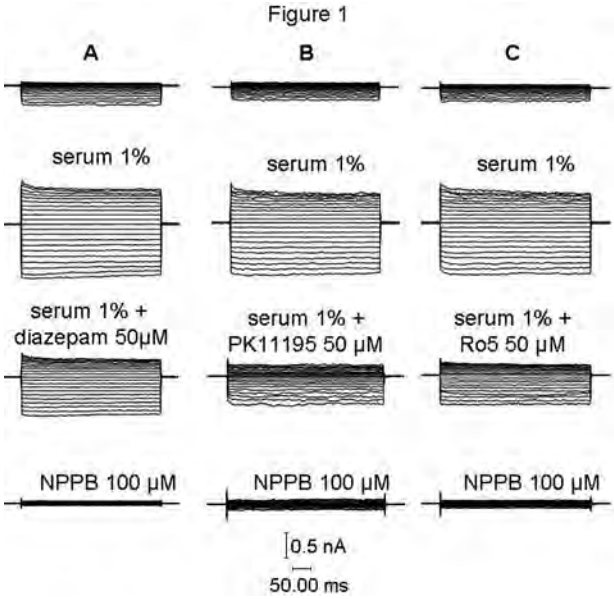

Figure 1 :Representative traces of effect of PBR ligands on whole cell current of malaria-infected human red blood cells.

Current were recorded using the whole-cell configuration on three malaria-infected human red blood cells(cell A, B and C), with sequential perfusion of bath solution.

First raw was recorded 5 min after obtaining whole-cell configuration. Second raw 15 min after 1% serum solution added to bath solution.

Third raw 15 min after perfusion with 1% serum + 50µM Diazepam(cell A), 50µM PK11195 (cell B) or 50 µM Ro5-4864 (cell C).

Fourth raw 5min after perfusion with 100µM NPPB.

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C14 and PC14

Electrolyte transport in the perfused *Drosophila* larval posterior midgut is selectively inhibited by paradoxical actions of fungal toxins.

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The *Drosophila* midgut is a powerful model for the study of function (1-3) and cellular homeostasis (4) of gastrointestinal epithelia. The posterior midgut secretes strong base into the lumen (1) driving it intensely alkaline (pH ~11). This is accomplished by secretion of $NaHCO_3$, and absorption of KCl and HCl into the haemolymph (2,3). One of the unique features of this epithelium is that the basal membrane (b) is an order of magnitude more resistive than the apical (a), and the overall transepithelial resistance (R_t) is very high (~1k$\Omega.cm^2$). This fortuitous situation enables excellent voltage-clamp not only of the epithelium, despite its tubular geometry, but also voltage-clamp of the basal membrane only (2). We used Nystatin, and the cyclic peptide Destruxin A, to elucidate transport pathways for electrolytes.

The midgut was perfused and superfused with a 1:3 mix of Schneider medium and Ringer. Intra- and extra-cellular ionic activities were measured with ion-selective microelectrodes. Luminal perfusion with 500 nM Nystatin in Schneider-Ringer did not show changes in transepithelial potential V_t (–42.4 ± 4.2 and –41.4 ± 4.3mV), transepithelial resistance R_t (781±58 and 786±128 $\Omega.cm^2$), and current Isc (47 ± 6 and 60 ± 13 $\mu A.cm^{-2}$), Vb (–62.3 ± 3.8 and –63.5 ± 6mV) and Va (20.1 ± 5.8 and 21.9 ± 8.1mV) ($p > 0.05$; $n = 10$). However the resistance ratio of basal to apical membranes was decreased from 4.2 ± 1.1 to 1.7 ± 2.1 ($n = 10$, $p < 0.03$).

Cyclic peptides like Destruxin A are highly toxic to diptera. We verified that non-selective channels (5-600 pS, n=30) were formed in artificial bilayer membranes (5), and expected that, as in the case of Nystatin, R_t would fall. Basal Destruxin A (200 μM) decreased V_t from –37.7 ± 2.5 to –8.3 ± 1.1 mV and Isc from 66.0 ± 9.0 to 10.0 ± 1.7 $\mu A.cm^{-2}$($n=17$; $p < 0.01$). R_t, however, *increased* from 664 ± 63 to 1062 ± 128 $\Omega.cm^2$($n=18$; $p < 0.01$) and depolarized apical (Va) and basal (Vb) membrane potentials (17.0 ± 3.2 mV to 7.4 ± 2.2 mV and –60.1 ± 3.9 to –17.0 ± 2.4 mV respectively ($n=18$, $p<0.01$). Rb/Ra ratios decreased (9.8 ± 1.7 to -3.8 ± 3.4 ($n = 17$, $p < 0.01$). The action of Destruxin A was dose-dependent; half maximal effective concentration was ~100 μM. For lower doses (≤ 50 μM) the recovery was very rapid (within 5 min) and both the basal (Vb) and apical (Va) membrane potentials hyperpolarized significantly after the washout of Destruxin A at these doses. Luminal Destruxin was without effect indicating that the effects of this toxin is probably through the tracheoles. The rise in R_t is probably due to the inactivation of channels in the apical membrane.

Data reported are means and S.E.M). All procedures accorded with current national legislation/guidelines and also current local and institutional guidelines.

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C15 and PC15

The function of CFTR high expresser cells in the possum proximal colon

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Although the possum proximal colon expresses the cystic fibrosis transmembrane conductance regulator (CFTR) (4) and the NaK2Cl cotransporter (NKCC1) (2), secretagogues do not stimulate electrogenic Cl^- secretion by this epithelium (3). Here we have investigated the function of CFTR in the possum proximal colon. CFTR was expressed at high levels in the proximal colon, but the CFTR immunoreactivity was restricted to a small population of randomly distributed cells in the surface epithelium and upper third of the crypts (n=3), reminiscent of the CFTR high expresser (CHE) cells seen in the intestine of eutherian mammals (1). Consistent with the absence of electrogenic Cl^- secretion the CHE cells do not express NKCC1, and while forskolin (20μM) stimulated an increase in short circuit current when the tissues were mounted in the Ussing chamber (n=20), this was not inhibited by bumetanide (100μM), although it was inhibited by mucosal N-(2-Naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide (GlyH101) (40μM) indicating it involved CFTR. Forskolin also stimulated a dose dependent (EC_{50}=1μM, n=7) increase in transepithelial resistance (R_T), which was dependent upon Cl^- (n=16). Furthermore, mucosal GlyH101 (40μM, n=6) increased R_T (ΔR_T=45±5 Ω cm^2, all values X±SEM) to a comparable extent as forskolin (20μM, ΔR_T=48±10 Ω cm^2), but the effects of forskolin and GlyH101 on R_T were not additive. Serosal DIDS (1mM) had a similar effect on R_T (ΔR_T=42±13 Ω cm^2, n= 6) and, as with mucosal GlyH101, the effects of serosal DIDS and forskolin were not additive. These data suggest that the CHE cells in the possum colon provide a transepithelial Cl^- conductance, consisting of CFTR in the apical membrane and a DIDS-sensitive anion conductance in the basolateral membrane, and this conductance is inhibited by forskolin. Measurement of the Cl^- current (I_{Cl}) in the presence of a mucosal to serosal gradient confirmed this proposal. When colonic epithelia were bathed in mucosal NaCl Ringer's and serosal NaGluconate Ringer's a I_{Cl} of 298±26μA cm^{-2} (n=8) developed, which was inhibited by forskolin with an IC_{50} (0.75μM, n=5) comparable to that for the increase in R_T in the absence of a gradient. Furthermore, mucosal GlyH101 (40μM, n=8) and serosal DIDS (1mM, n=8) inhibited 82±4% and 89±4% of the I_{Cl}, respectively, whereas serosal GlyH101 and mucosal DIDS had no effect. Thus, in the possum proximal colon CHE cells provide a transepithelial Cl^- conduc-

tance and, as absorption in this tissue is driven by electrogenic Na^+ transport, it is likely that this is an important route for the passive absorption of Cl^-. Inhibition of this conductance by forskolin would reduce Na^+ absorption by restricting the movement of Cl^-.

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C16 and PC16

New insight into CLC-2 chloride channel function: GlialCAM, a protein defective in a leukodystrophy is a channel subunit in glial cells

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CLC-2 is an ubiquitously expressed inwardly rectifying chloride channel with many candidate functions. Brains of Clcn2 $^{-/-}$ mice show vacuole formation in myelin sheets[1]. Similar vacuolar structures were found in patients which carried mutations in MLC1, a transmembrane protein of unknown function, leading to a rare leukodystrophy called MLC (megaencephalic encephalopathy with subcortical cysts) [2]. Since MLC1 does not interact with CLC-2 and no mutations in the CLCN2 gene were found in MLC patients [3], a role of CLC-2 in MLC seemed to be excluded. Recently a second disease gene, GlialCAM, causing MLC was found [4]. GlialCAM is a cell adhesion molecule mainly expressed in glial cells [5] and interacts directly with MLC1 [4].

Our biochemical approach to identify interacting partners for GlialCAM uncovered CLC-2 as a major binding partner besides MLC1 in brain. CLC-2 and GlialCAM co-localise in Bergmann glia, myelin and at astrocyte-astrocyte contacts at the endfeet. In HEK cells, we observe a shift of CLC-2 being uniformly distributed over the plasma membrane to areas of cell contacts when co-expressed with GlialCAM. Since GlialCAM alone is localised in cell contacts as well, it is likely that it acts as a carrier molecule for CLC-2. Functionally, co-expression of CLC-2 with GlialCAM resulted in a large increase of CLC-2 mediated currents in Xenopus oocytes and HEK cells. The

activation of the channel becomes almost instantaneous and currents lose rectification. CLC-2/GlialCAM currents resemble those of an N-terminal deletion mutant of CLC-2 (ΔN). However, in contrast to ΔN the CLC-2/GlialCAM complex stays osmosensitive and reduces pH blockage at acidic conditions. Anion selectivity and Cd^{2+} block remain unchanged in the complex. We studied four disease causing GlialCAM mutations in the IgV domain. All of them disrupted the clustering of CLC-2/GlialCAM at cell contacts, but did not alter the ability to increase and modify CLC-2 mediated currents.
In conclusion, we propose that CLC-2 is localised in cell contacts in glial cells and modulated through an interaction with the common gate by the cell adhesion molecule GlialCAM. This interaction may be important in the pathology of MLC disease and opens new insights into the role of CLC-2 in glial cells.

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C17 and PC17

Effects of flavonoids on agonist-stimulated chloride secretion in Caco-2 cells and mouse colon

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The flavonoids quercetin, kaempferol and genistein are present in the diet and known to stimulate chloride secretion in the intestine through a cAMP-dependent pathway (Cermak *et al* 2001). However, many such *in vitro* studies have used concentrations of the aglycone form of the flavonoid well in excess of the bioavailable concentrations observed in plasma after a meal. We therefore tested the activity of these flavonoids on colonic chloride secretion at concentrations potentially achievable in vivo (<30 μM serosal, <100 μM mucosal). The possible augmentation of secretory responses to the agonists ATP and forskolin was also studied. Caco-2 human colonic epithelial monolayers and isolated mouse distal colonic mucosae were mounted in Ussing chambers and changes to the short-circuit current (ΔISC) were measured in response to flavonoids and other agents. Values of ISC are pre-

sented as mean ± SD (n). Differences between means were analysed by t-test or one-way ANOVA with Dunnett's post-test and deemed significant at $p<0.05$. mAll three flavonoids stimulated a transient increase in chloride secretion in Caco-2 monolayers when added to the mucosal side. The EC50 for the response to quercetin was 30 μM with a ΔISC of 8.82 ±3.98 μA/cm^2, n=6. ATP (100 μM) stimulated similar increases of ISC from the mucosal side (8.29 ± 2.24 μA/cm^2, n=8) and these responses were significantly increased by pre-treatment with quercetin 100 μM or kaempferol 100 μM to 17.04 ± 7.00 μA/cm^2, n=7, and 15.68 ± 4.16 μA/cm^2, n=4, respectively. Serosal ATP responses were smaller (1.84 ± 1.11 μA/cm^2, n=5) but significantly increased by mucosal addition of quercetin 30 μM (39.15 ± 20.5 μA/cm^2, n=6), and genistein 30 μM (76.34 ± 17.23 μA/cm^2, n=3). MDL12330A, an adenylyl cyclase inhibitor, abolished the responses to quercetin or kaempferol, but not the Ca^{2+}-dependent secretagogue ATP, indicating stimulation of cAMP signalling by the flavonoids. In mouse colon, genistein was effective on the mucosal side in the range 1-100 μM, but the response to serosal ATP was not augmented. However, the lowest concentration of genistein (1 μM), which alone evoked only a small ΔISC (<3 μA/cm^2) significantly augmented secretory responses to low concentrations of forksolin: at 0.1 μM, from 25.10 ± 26.95 to 87.65 ± 54.31 μA/cm^2, n=11, and at 0.3 μM from 44.10 ± 39.8 to 106.50 ± 56.64 μA/cm^2, n=7. Responses to forskolin at 1-10 μM were not augmented. We suggest that genistein at a likely bioavailable concentration may interact with cAMP-dependent agonists to enhance chloride secretion in the colon. The consequence may include a natural secretory laxative effect in the gut.

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C18 and PC18

The apical Na^+/H^+ exchanger NHE3 interacting PDZ protein NHERF2 is strongly lipid raft-associated and determines the raft association of NHE3 in murine small intestine

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Backround: Na^+/H^+ exchangers play a major role in the regulation of intracellular and intravascular volume and maintain it within a physiological range. Na^+/H^+ exchanger 3 (NHE3) is expressed on the apical membrane of intestinal epithelia and is scaffolded by Na^+/H^+ exchanger regulatory factor (NHERF) family of PDZ-adapter proteins which are also involved in its regulation and membrane traffick-

ing. It has been demonstrated that a population of NHE3 resides in lipid rafts of the Brush Border Membrane (BBM). Similar to the role of the NHERFs, lipid rafts are also considered to be platforms enabling protein-protein interaction and signal transduction. **Aim**: In this study, we asked the question whether the NHERFs are differentially associated with the raft and non-raft fraction of NHE3, and if the presence or absence of the NHERFs influences the raft vs. non-raft distribution of NHE3, and its transport activity. **Methods and Results**: Murine BBMs were isolated by the divalent cation precipitation technique, and lipid rafts were subsequently separated by Triton X-100 membrane solubilisation and flotation of detergent-resistant membranes in an OptiPrep density gradient. NHE3 was found to be partially lipid raft associated. Interestingly, NHERF2 was found to be strongly lipid-raft associated, NHERF1 partially, and NHERF3 was exclusively in the non-raft fraction. In the absence of NHERF2 expression, the percentage of raft-associated NHE3 was strongly decreased, and NHE3 function, measured both in isolated BCECF-loaded small intestinal villi by pHi-fluorometry and in luminally perfused mice in vivo, was altered, as was NHE3 trafficking within the Brush border membrane. **Conclusions**: The data demonstrate that in the Brush border membrane of murine small intestine, NHE3 differentially associates with the different NHERFs in the raft and non-raft fraction, with NHERF2 being strongly associated with raft-associated NHE3. NHERF2 deletion resulted in loss of raft-associated NHE3 as well as alteration of NHE3 transport function. This suggests that PDZ-scaffolding proteins are involved in the retention of membrane proteins within lipid raft platforms.

Na+-H+ exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal brush border: a role for rafts in trafficking and rapid stimulation of NHE3

Li X, Galli T, Leu S, Wade JB, Weinman EJ, Leung G, Cheong A, Louvard D, Donowitz M.

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C19 and PC19

Dependence of CFTR Trafficking on LMTK2 kinase activity in Human Colonic Epithelial and Mouse Primary Airway Epithelial Cells

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The magnitude of transepithelial chloride transport depends on both the activation status (i.e. phosphorylation) and number of CFTR channels present in the apical membrane. In polarized epithelia, CFTR undergoes rapid and efficient endocytosis with subsequent recycling back to the plasma membrane. The molecular mechanisms underlying CFTR recycling are poorly understood, yet this critical process is required for the appropriate channel copy number at the apical membrane, and is defective in the common CFTR mutant ΔF508. Here we examined

the role of the novel kinase LMTK2 in regulating the trafficking of CFTR in a human colonic epithelial cell line that endogenously expresses both CFTR and LMTK2 (T84), as well as in primary mouse tracheal epithelial cells derived from wt (LMTK2+/+) and LMTK2-/- knockout mice. RNAi-mediated knockdown of LMTK2 had no effect on CFTR endocytosis in T84 cells, but markedly inhibited recycling of internalized channels, leading to a net reduction in cell surface protein and a concomitant reduction in cAMP activated chloride current. In contrast, over expression of LMTK2 increased CFTR recycling, with an attendant increase in CFTR copy number at the apical membrane and increased transepithelial chloride secretion. Expression of a kinase-dead LMTK2 mutant acted as a partial dominant-negative construct, implicating a phosphorylation event as a key element in the LMTK2 dependent recycling of CFTR in human polarized epithelia. Indeed LMTK2 is able to phosphorylate Ser737 in CFTR's Regulatory domain. To validate further BREK as a regulator of CFTR trafficking, we utilized MTE cells obtained from wild-type and LMTK2 knockout mice as an animal model. Loss of LMTK2 activity was not associated with any loss of transepithelial resistance, indicating that the integrity of the epithelial monolayer was not compromised. However, forskolin stimulated short circuit currents were markedly lower in MTE from LMTK2-/- animals compared to those derived from wild-type animals, essentially recapitulating the observations derived from T84 cells. Peptide array analysis argues that CFTR is the primary substrate for LMTK2 dependent phosphorylation, indicating the unique position of LMTK2 as a novel drugable target to enhance the recycling of mutant CFTR back to the cell surface.

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PC20

Disruption of clc-5 leads to a redistribution of annexin A2 in cells of the collecting duct and distal convoluted tubule of a Dent's disease mouse model

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Mutations in CLCN5, encoding the voltage-dependent Cl^-/H^+antiporter, CLC-5, cause Dent's disease (Type 1) characterised by low molecular weight proteinuria, hypercalciuria, and nephrolithiasis. Impairment of endocytosis occurs primarily in the proximal tubule, but CLC-5 is also expressed more distally in the collecting duct (CD) intercalated cells[1]. Expression of clc-5 occurs in several distal/collecting duct mouse cell lines such as mIMCD-3 and mpkDCT cells[2,3]. In mIMCD-3 cells, clc-5 ablation results in defective endocytosis and plasma membrane expression of the crystal adhesion molecule, annexin A2 together with crystal agglomeration[3]. Antisense clc-5 treatment of the mpkDCT cell model of the distal convoluted tubule (DCT) disrupts endocytosis[2,4]. Here we investigate the effect of clc-5 disruption on annexin A2 distribution in mpkDCT cells. We extend this to examine annexin A2 localisation in the CD and DCT of the Guggino clcn5 knockout mouse, a model that recapitulates the renal attributes of Dent's disease, including intra-tubular Ca^{2+}-crystal deposition[5].

Using immunocytochemistry and confocal microscopy, annexin A2 distribution was determined in mpkDCT cells transfected with control GFP (transfection marker) and in cells where endogenous clc-5 was disrupted through cotransfection with antisense clc-5 and GFP[2,3]. Paraffin embedded sections of kidney tissue of wild type (WT) and clcn5 knockout (KO) mice (12 months, high citrate diet) were subjected to antigen retrieval with 0.8M urea and assessed for annexin A2 distribution. Annexin A2 was detected by a rabbit anti-annexin A2 antibody (H-50 raised against residues 1–50 of human protein, cross-reactive with mouse) at 1:100 (Santa Cruz). Goat anti-aquaporin 2 antibody (epitope mapping at C-terminus, human origin, cross-reactive with mouse, 1:250) and goat anti-calbindin antibody (epitope mapping at C-terminus of Calbindin D28K, human origin, cross-reactive with mouse,1:100) were used as markers of the CD and DCT respectively. Appropriate secondary antibodies were used.

Control GFP transfected mpkDCT cells showed an intracellular perinuclear location for annexin A2 (n=6). Following transfection with antisense clc-5, there was a marked redistribution of cytoplasmic annexin A2 to the cell periphery (n=5). In the WT mouse, annexin A2 showed an intracellular, vesicular pattern in cell types within the DCT and CD. In the clcn5 knockout, annexin A2 relocalised to the apical (lumen) cell pole in cells within the DCT and CD. All experiments were carried out in material from 3 KO or WT animals. We speculate that abnormal expression of the crys-

tal binding molecule annexin A2 at the cell surface, together with hypercalciuria, will facilitate intra-tubular Ca^{2+}-crystal retention within the collecting ducts of KO animals.

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work supported by KRUK & NCKRF

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PC21

Investigation of UT-B urea transporter isoforms in the human gastrointestinal tract

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The symbiotic relationship that exists between humans and their intestinal bacteria is crucial in maintaining our gastrointestinal health. UT-B urea transporters facilitate the entry of urea into the lumen of the gastrointestinal tract and hence play a significant role in the urea nitrogen salvaging mechanism believed to support bacterial growth. Previously, using an antibody raised against bovine UT-B1, we detected UT-B proteins in human colonic mucosa in the form of 30kDa unglycosylated and 35kDa glycosylated signals. In contrast, a study by Inoue et al. utilising a different antibody detected a glycosylated 50kDa UT-B protein (2), suggesting there may be more than one isoform present in the human colon. To determine whether different UT-B isoforms are present in the human gastrointestinal tract, we produced and characterised a new UT-B antibody that was raised against the C-terminal of human UT-B1. Using western blotting techniques, we detected multiple protein signals of different sizes - namely 34, 38, 43 and 55kDa. These proteins were present in stomach, small intestine and colonic tissue (obtained from surgical resection procedures, with consent), as well as the Caco-2 intestinal cell line. Although pre-incubation with immunizing peptide completely prevented these signals, surprisingly none of them represented glycosylated proteins. Preliminary RT-PCR investigation of human colonic cDNA confirmed the presence of more than one human UT-B transcript (e.g. both UT-B1 and UT-B2 detected). Together, these data suggest the existence of multiple UT-B isoforms throughout the human gastrointestinal tract. Further studies are now required to determine the precise nature, cellular localization and function of these different gastrointestinal human UT-B urea transporters.

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PC22

Intermittent fasting reduces placental Ca^{2+}-ATPase expression in the rat

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During mammalian gestation the fetus is dependent upon the placenta for the transfer of maternal nutrients. In humans placental growth responds to maternal influences such as diet and physical activity. While the effects of dieting on placental development and function have been studied widely, the effects of intermittent fasting have not. During the month of Ramadan Muslims refrain from eating and drinking during the hours of daylight. Although pregnant women are exempt from the fast many participate. In a recent human study conducted in Saudi Arabia we showed that babies who were in the second or third trimester of gestation when their mothers fasted had lower mean placental weights and placental weight to birth weight ratios compared with those not in utero during Ramadan [1]. We observed similar changes in placental and body weights in rats whose mothers were subject to intermittent fasting (IF) during pregnancy [2]. Approximately 80% of the calcium forming the fetal skeleton is transported across the placenta from the mother by the end of gestation. Disruptions to calcium homeostasis during gestation can lead to an increased risk of developing osteoporosis in later life [3]. Calcium transport across placental epithelium occurs in 3 stages: diffusion into the trophoblast from maternal plasma through epithelial Ca2+ channels of the transient receptor potential (TRP) gene family, transfer across the trophoblast cytoplasm bound to the calcium binding protein calbindin-D9K and lastly active extrusion into the fetal compartment via the plasma membrane Ca2+-ATPase (PMCA) localised to the fetal facing basal plasma membrane [4]. The aim of this study was to determine whether maternal IF alters active placental Ca2+ transport. Control pregnant Wistar rats had access to food ad libitum (C n=6); in rats subject to intermittent fasting food was withdrawn at 17.00 and returned at 09.00 daily from day 1 of pregnancy until birth; food was available ad libitum for the remainder of the day (e.g. from 09.00 to 17.00) (IF n=6). All rats had free access to water throughout. Placental tissue was harvested at embryonic day 21 and PMCA expression determined by Western blot. PMCA expression in IF placentas was significantly lower compared with that in controls ($P<0.05$, Mann-Whitney test, Figure 1). These

data suggest that fetal Ca2+ accretion is diminished in the later stages of gestation exposed to intermittent fasting.

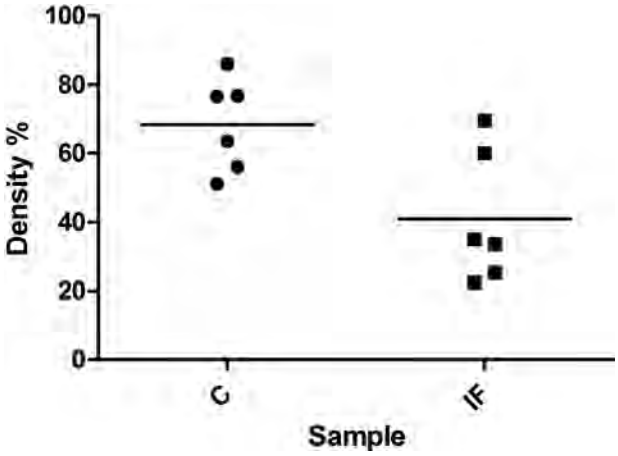

Figure 1 PMCA expression in placenta of control and IF rats, shown as density % of beta actin (n=6). P<0.05.

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2. Wilkinson RJ et al (2010). Proc Physiol Soc 19, PC244

3. Tobias JH & Cooper C (2004). J Bone Miner Res 19, 177-182

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PC23

Expression of the ABC transport proteins MDR1 (ABCB1) and BCRP (ABCG2) in bovine rumen

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Since plant vacuoles are the cellular repository for detoxification of heavy metal conjugates, herbicides and toxins (Rea et al, 1998), mastication and rumen digestion of plant based forage in bovines will release accumulated toxins within the rumen which is also the major site for absorption of short-chain fatty acids from microbial fermentation (Graham and Simmons 2005). Accordingly we have investigated the ruminal expression of bovine ABC transporters associated with cyto-

protection of gastro-intestinal enterocytes against xenobiotic exposure, namely MDR1 (ABCB1), MRP2 (ABCC2) and BCRP (ABCG2) (Haslam et al, 2008).
Bovine rumen samples from the ventral sac were obtained post-mortem from a commercial slaughterhouse after humane killing. Epithelial tissue was then stripped from the underlying muscle layers and samples prepared for immunohistochemistry, SDS-PAGE/ Western blotting and total RNA isolation for RT-PCR as previously described (Graham and Simmons 2005). Bovine-specific primers used for PCR were designed for MDR1, BCRP and MRP. β-actin was used as a control transcript. Using 30 cycles PCR products of the predicted size were observed for both MDR1 and BCRP, but not for MRP2. Figure 1A shows a Western blot of MDR1 expression using C219 primary monoclonal antibody (mab) in bovine rumen with a doublet at an apparent molecular size of ~ 170-180 kD. Immunolocalisation of MDR1 using UIC2 mab within cryosections of bovine rumen confirms MDR1 expression. The tissue was co-stained with the nucleic acid marker ethidium homodimer 1 with the overlay combining MDR1/nucleic acid staining. MDR1 expression is absent from outer stratum corneum but extensive membrane staining is observed in cells of the stratum granulosum, stratum spinosum and stratum basale. Protein expression and immunolocalisation was also confirmed for BCRP, with prevalent staining in the stratum basale, becoming weaker in the stratum spinosum and stratum granulosum (not shown). No MRP2 expression was apparent as confirmed by RT-PCR, immunostaining and western blot.
We conclude that extensive expression of both MDR1 and BCRP within bovine rumen may play a role in cytoprotection of ruminal epithelium against xenobiotics present in forage.

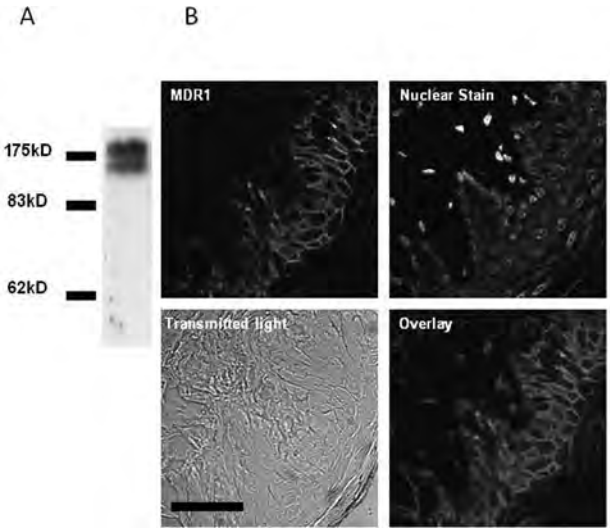

Figure 1. A) Western blot of MDR1 expression using C219 mab, and B) immunolocalisation of MDR1 expression within bovine rumen using UIC2 mab. Scale bar = 50μm.

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PC24

Cross species mapping of cAMP response element, and supporting transcription factor binding sites, in promoter regions of genes involved in regulating ion and water transport in the cortical collecting duct

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Aldosterone and vasopressin play key roles in the temporal regulation of an increasingly large network of proteins involved in the fine tuning of sodium and water reabsorption in the distal nephron. In particular, vasopressin has been shown to trancriptionally regulate the expression of proteins involved in both processes through elevation of cAMP that ultimately results in the activation of the transcription factor cAMP response element binding protein (CREB). CREB can then bind to cAMP response element (CRE) regions within the promoter region of target genes to induce or enhance transcription. Here we have mapped CRE and additional regulatory sites within a 4Kb promoter region of 29 proteins involved in controlling ion and water absorption in the collecting duct with the aim of establishing the prevalence, conservation among species and potential functional importance of this regulatory sequence in the human sequences.

CREs are present in all human promoter sequences of the 29 genes of interest and show varying degrees of conservation with other species. We mapped single nucleotide polymorphisms (SNPs) within CREs in SCNN1A, B and G. One of the two SNPs in SCNN1G occurring in a non-conserved site and is associated with a predisposition to hypertension. SNPs within CREs were also found in human promoters for aquaporin water channels, Na+/K+ATPase subunits and protease enzymes.

We conclude that the previously described SNP (-173A/G; Iwai et al., 2001) exists in a CRE site and that the additional SNPs we have mapped are worthy of future in vitro study to examine their affects on vasopressin mediated gene transcription.

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PC25

The anion exchanger Slc26a6 functionally interacts and tethers the cytoplasmic carbonic anhydrase II to the apical membrane in murine duodenum

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Background and Aims: Slc26a3 and a6 are members of a multi-anion transporting family and both mediate Cl-/HCO3- exchange. Both play important roles in intestinal HCO3- secretion and electroneutral salt absorption, but only Slc26a6 can either export or import HCO3- depending on pHi. In addition, only Scl26a6 has a carbonic anhydrase (CA) binding motif. CAs catalyze the reversible conversion of CO2 to HCO3-, and some anion transporters form transport metabolon with CAs, to maximize the coupled catalytic/transport flux. The aim of this study was to find evidence for functional coupling and co-localization of Slc26a3 and a6 with CAII in vivo.

Methods and Results: CAII was found to co-localize with Slc26a6 in the apical membrane of villous enterocytes by immunohistochemistry in WT and Slc26a3 KO mice, while it had a cytoplasmic distribution in Slc26a6-deficient villi. Further, CAII could be co-immunoprecipitated with Slc26a6 from the native intestine. The amount of total CAII was reduced in Slc26a6-deficient duodenum as shown by western blot. The proximal duodenum of isoflurane-anesthetized (2% in 10-15% O_2 and 85-90% air) Slc26a3, a6, CAII KO, or double KO and WT mice was perfused in situ, and basal and PGE2-stimulated HCO3- secretion was determined by back titration. Deletion of Slc26a6 slightly reduced both the basal rate and more so the PGE2-stimulated response, and this reduction was dramatically augmented by additional CAII ablation, which, by itself, did not have a significant effect. This suggests that the tethering of CAII to the apical membrane by Slc26a6 does not only affect transport of HCO3- via Slc26a6, but also via other HCO3- transporters such as CFTR or Slc26a3. Similar results were obtained by luminal application of the carbonic anhydrase inhibitor methazolamide (MTZ). Slc26a3 KO duodenum displayed reduced basal HCO3- secretory rate, and this difference was abolished by MTZ application. Slc26a3 KO mice had normal HCO3- response to PGE2 stimulation.

Conclusion: The anion exchanger Slc26a6, which is strongly expressed in the brush border membrane of the upper intestine, serves as an apical membrane anchor for CAII. This appears to not only serve for HCO3- transport via Slc26a6 but also via other apical membrane HCO3- transporters Functional augmentation of Slc26a6 by CAII during HCO3- secretion in the murine duodenum in vivo may explain in part the strong dependency of Slc26a6 HCO3- export function on the blood acid/base parameters.

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PC26

The effect of azithromycin on rat colonic epithelium

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The mechanisms of antibiotic adverse effects on gastrointestinal tract functions are not clear. The macrolide antibiotics have wide spectrum of nonantibiotic action. It was shown that they can directly influence on airway epithelium ion transport. In present study we tested the hypothesis that treatment with 15-member macrolide antibiotic azythromycin may affect net water and electrolytes movement across colonic epithelium.
Methods: The study was done on male Wistar rats (180-230 g). Azithromycin (15 mg/kg, per.os.) was given orally each day for 5 days. Net water and ion trasport (Na, K, Cl) were evaluated on the 6th day by isolated colonic loop perfusion technique in vivo on anaesthetized rats (urethane, 1.1 g/kg intraperitoneal). The colonic levels of malondialdehyde and the catalase activities by colorimetric assays and superoxide dismutase activity by zymograthy were measured. Blood was obtained by cardiac puncture after terminal CO_2 treatment. The blood lipids concentration was evaluated by gas-liquid chromatography. The fecal microflora has been analyzed by bacteriological culture methods.
Results: Treatment with azithromycin increased water 2.5-fold ($p<0.001$), sodium 1.7-fold ($p<0.001$), chloride absorption 1.3-fold ($p<0.05$) and decreased potassium secretion ($p<0.05$). This effect was associated with 2-fold increase growth of Staphylococcus epidermidis, Streptococcus and Candida, while levels of Bifidobacterium and Lactobacillus were unchanged. We found increased catalase ($p<0.01$) and superoxide dismutase ($p<0.05$) activity in colonic mucosa after 5 days of azithromycin treatment, while levels of malondialdehyde (the intensity of lipid peroxidation) did not change. Azithromycin did not affect levels of polyunsaturated and unsaturated fatty acids concentration in whole blood of rats.
Conclusions: 1) Azithromycin directly affects ion and water transport across colonic epithelium; 2) Therapy with azithromycin evoke pathogenic microflora overgrowth that was followed by oxidative stress in colonic epithelium.

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PC27

Refining an haemolysis assay for diagnosis and prognosis of sickle cell disease

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Sickle cell disease (SCD) is one of the commonest severe inherited disorders. All SCD patients have the abnormal haemoglobin HbS in their red blood cells (RBCs) instead of the normal adult HbA. Deoxygenated HbS polymerises and distorts RBCs into bizarre shapes. The resulting pathology is extensive, with anaemia and vaso-occlusive episodes of pain, organ damage and mortality. Early diagnosis is critical to manage the severest complications of SCD [3]. RBCs from SCD patients have a high cation permeability causing solute loss, shrinkage and raised [HbS]. As the polymerisation lag time is inversely proportional to a very high power of [HbS], modest shrinkage is sufficient to encourage greatly HbS polymerisation and thereby contribute to disease. Several pathways participate in the increased permeability [2]: KCl cotransport, the Gardos or Ca^{2+}-activated K^+ channel and a deoxygenation-induced non-selective cation pathway (P_{sickle}). P_{sickle} has a central role mediating Ca^{2+} entry, activating the Gardos channel and causing other sequelae such as phosphatidylserine exposure. Although the principle defect is increased cation permeability, deoxygenated RBCs from SCD patients have been shown to haemolyse in isosmotic non-electrolyte solutions [1]. Lysis is accompanied by entry of non-electrolyte and a number of features suggestive of P_{sickle} involvement [1]. As P_{sickle} is only activated in RBCs from SCD patients, this phenomenon may be used to design a simple diagnostic test for SCD which might be prognostically useful. Here we further characterise this assay. RBCs (from discarded samples) were washed in MOPS-buffered saline (in mM: NaCl 145, glucose 5, MOPS 10, pH 7.4, 290±5mOsm.kg^{-1}, 37ºC unless otherwise stated) and re-suspended in isosmotic sucrose solution (sucrose replacing NaCl). At pH 7.4, RBCs from HbSS and HbSC SCD patients lysed on deoxygenation; those from HbAA and HbAS individuals did not (Fig 1a). Ouabain (100μM), bumetanide (10μM), or raising or lowering [sucrose], had minimal effect on lysis. Clotrimazole (10μM) or EGTA (2mM) modestly, but repeatably, increased lysis. Deoxygenation in the presence of *o*-vanillin (5mM) to prevent HbS polymerisation abrogated haemolysis. Effect of pH was studied. At pH 6, RBCs from SCD patients lysed even when oxygenated, an effect also prevented by *o*-vanillin (Fig 1b). Findings show that haemolysis in isosmotic non-electrolyte solutions appears exclusive to RBCs from SCD patients. Gardos channel activation and KCl loss reduces lysis by only a small extent. Hypo- or hypertonicity to alter RBC volume or chemical driving force for sucrose entry is without effect. The inhibitory effect of *o*-vanillin is consistent with HbS polymerisation being a pre-requisite for

lysis. The ability to use oxygenated solutions at low pH will facilitate development of the test.

Fig. 1a - Haemolysis of deoxygenated RBCs in sucrose buffer at pH 7.4: HbAA, HbAS, HbSC and HbSS genotypes. Data represent means ± SEM.

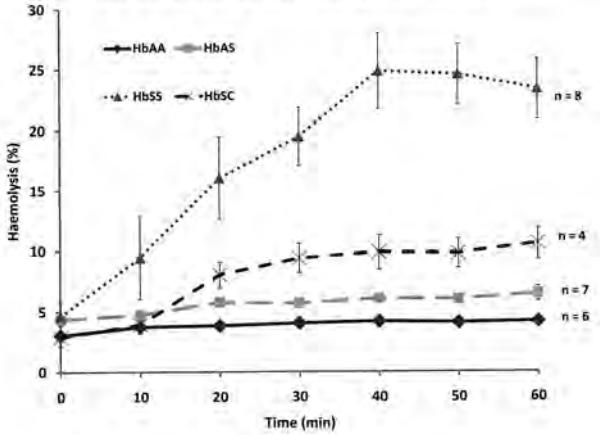
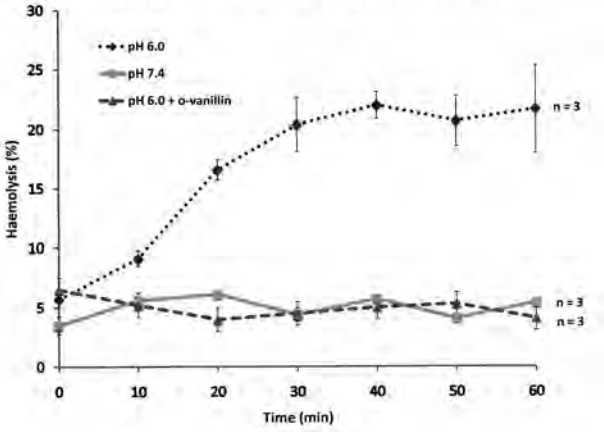


Fig. 1b - Haemolysis of oxygenated HbSS RBCs in sucrose buffer at pH 6.0, 7.4 and pH 6.0 with 5 mM *o*-vanillin. Data represent means ± SEM.



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Lew, V. L. & Bookchin, R. B. (2005). *Physiol. Rev.* **85**, 179-200.

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PC28

Effect of treatment with hydroxyurea on red blood cell phenotype in patients with sickle cell disease

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Red blood cells (RBCs) from patients with sickle cell disease (SCD) contain the abnormal haemoglobin HbS instead of the normal adult HbA [4]. Deoxygenated HbS polymerises, distorts RBCs into bizarre shapes and alters rheology. The resulting pathology is extensive but treatment remains largely supportive. Severely affected individuals, however, are sometimes given hydroxyurea which ameliorates SCD complications probably by encouraging expression of fetal Hb, HbF. RBCs from SCD patients show high cation permeability which causes solute loss, shrinkage and increased [HbS]. As the lag time to polymerisation is inversely proportional to a very high power of [HbS], modest shrinkage is sufficient to encourage greatly HbS polymerisation and thereby contributes to disease. Several pathways participate in the increased permeability [3]: KCl cotransport (KCC), the Gardos or Ca^{2+}-activated K^+ channel and a deoxygenation-induced non-selective cation pathway (P_{sickle}). P_{sickle} mediates Ca^{2+} entry, activating the Gardos channel and causing phospholipid scrambling. There is little information on how hydroxyurea alters these parameters [1]. Routine discarded blood samples were obtained from SCD patients using EDTA as anticoagulant. RBCs were washed in saline comprising (in mM) NaCl 145, glucose 5, MOPS 10, pH 7.4, 290±5mOsm.kg^{-1}, 37ºC (unless stated otherwise). O_2 tension was controlled using Eschweiler tonometry and a Wösthoff gas mixer. The activity of the main transport pathways were measured using $^{86}Rb^+$ as a K^+ congener (KCC as the Cl^--dependent flux replacing Cl^- with NO_3^-, Gardos channel as the clotrimazole (CLT 5μM)-sensitive flux, P_{sickle} as Cl^--independent, CLT-insensitive flux) [2]. Ouabain and bumetanide were present to inhibit the Na^+/K^+ pump and Na^+-K^+-2Cl^- cotransporter. Phosphatidylserine (PS) exposure was measured using FITC-lactadherin by FACS. Haemolysis was measured in isosmotic sucrose solution (salts replaced with sucrose). Cell morphology was assessed under light microscopy after fixing in saline plus 0.3% glutaraldehyde. Compared to untreated SCD patients, RBCs in those given hydroxyurea showed a reduced percentage of sickled cells on deoxygenation (Fig 1a). P_{sickle}, Gardos channel and KCC activtiy were also all inhibited (Fig 1a & 1b). In addition, KCC showed greater O2 dependence (Fig 1b). RBCs were also protected by about 50% against haemolysis in isosmotic sucrose solution. Finally, deoxygenation-induced PS exposure appeared unaffected. Findings increase our understanding of how treatment with hydroxyurea, and increased expression of HbF, protect RBCs from SCD patients from some of the deleterious

consequences of the presence of HbS. It is possible that some of these RBC phenotypes are involved in amelioration of the complications of SCD.

Fig 1a: Activity of P_{sickle} in mmol.(l cells.h)$^{-1}$ and morphological sickling as % total at different O_2 tensions (from 150-0mmHg) in RBCs from SCD patients treated without or with hydroxyurea (HU), means ± S.E.M., n = 5.

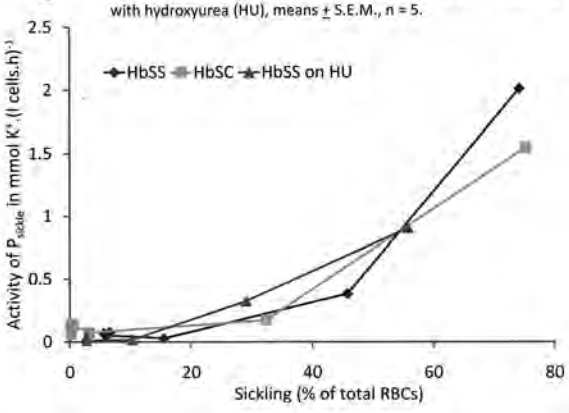
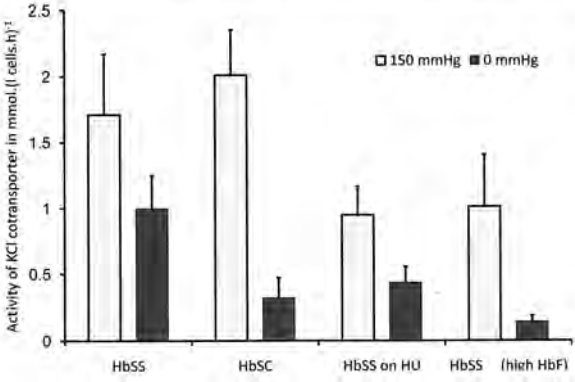


Fig 1b: Activity of KCl cotransporter in mmol.(l cells.h)$^{-1}$ at 150 or 0mmHg O_2 tension in RBCs from SCD patients treated without or with hydroxyurea (HU), means ± S.E.M., n = 5.



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PC29

Voltage-induced Na^+ and K^+ currents in human embryonic kidney epithelial (HEK293) cells

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The biophysical properties of cloned ion channels are often studied by characterising the conductance conferred upon HEK293 cells by heterologous expression of wild type / mutant channel protein. Whilst this approach clearly depends upon the assumption that HEK293 cells do not express any significant background conductance, voltage-gated ion channels have been described in these cells (e.g. Moran *et al*. 2000; Smirnov *et al*., 2003; He & Soderland, 2010). Since these endogenous conductances have the potential to complicate studies of cloned channels, the aim of the present study was to characterised the voltage-gated conductances present in this laboratory's stocks of HEK293 cells in order to facilitate the design of subsequent studies of heterologously expressed channels.

Analysis of currents recorded from cells held under voltage clamp (whole cell configuration) using pipette and bath solutions designed to maintain quasi-physiological ionic conditions ($[Na^+]_o$ = 144 mM; $[K^+]_i$ = 113 mM) showed that depolarisations (Fig. 1Aa) to potentials >-50 mV evoked outward currents that developed over ~20 ms (Fig 1Ab). Iso-osmotically replacing bath Na^+ with K^+ had little effect upon these outward currents but did evoke a transient "tail current" that became apparent when V_m was repolarised (I_{Tail}, Fig. 1Ac). Experiments in which the K^+ content of Na^+ free bath solutions was varied by iso-osmotically substituting N-methyl-D-glucammonium ($NMDG^+$) showed that I_{Tail} always reversed at a potential essentially identical to E_K (Fig 1B). Moreover, replacing pipette K^+ with Cs^+ consistently (n = 7) abolished the depolarization-induced outward current. Further examination of currents recorded under standard conditions (Fig 1Ab) showed this voltage-induced outward current was preceded by a transient (~10 ms) inward current that persisted after internal K^+ had been replaced by Cs^+ (n = 7). This inward current became evident once V_m was depolarized past -50 mV, and reversed at a potential essentially identical to E_{Na}. Moreover, its magnitude was greatly (~95%) reduced if bath Na+ was replaced with $NMDG^+$ (Fig. 1C).

HEK293 cells thus express voltage-gated K^+ and Na^+ channels that allow depolarizing voltage pulses to evoke a transient, inward Na^+ current that is succeeded by a sustained outward K^+ current. Although derived from the human kidney, it is therefore clear that HEK293 cells display a phenotype reminiscent of a neurone rather than an absorptive epithelial cell. Moreover, the presence of these voltage-gated conductances has the potential to interfere with the characterisation of cloned channels expressed in these cells.

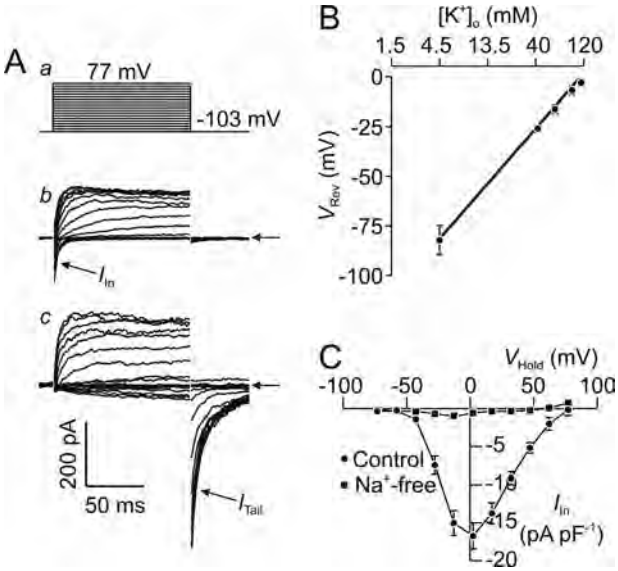

Fig. 1. (A) Currents evoked by depolarizing pulses (a) were recorded (n = 5) under standard conditions (b) and after bath Na^+ had been replaced by K^+ (c). Arrows indicate the zero current. (B) Cells were depolarized to 67 mV (50 ms) at different values of $[K^+]_o$ and the reversal potential (V_{Rev}) for I_{Tail} (see Ac) determined and plotted (n = 4) against $[K^+]_o$; the solid line shows the ideal relationship (Nernst equation) for a K^+ conductance. (C) Relationship between V_{Hold} and the peak inward current (I_{In}, see Ac) measured using Cs^+-rich pipette solutions. Data were recorded (n = 7) under control conditions and after bath Na^+ had been replaced by $NMDG^+$. All data are as mean s.e.m.

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PC30

A cautionary tale regarding preparation of intestinal brush border membrane for detection of GLUT2 by Western blotting

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GLUT2 expression at the jejunal brush border membrane (BBM), particularly under conditions of increased luminal glucose concentration or in experimental diabetes

mellitus (DM) has been previously demonstrated (Kellett & Helliwell et al 2000, Au et al 2002). However some studies have failed to detect expression of GLUT2 at the BBM, implying that glucose uptake across this membrane is exclusively SGLT1-mediated (Cui et al 2005, Batchelor et al 2011). Our present work provides a methodological explanation for the failure to detect GLUT2 at the BBM by western blotting.

GK rats (a model of type 2 DM) and Wistar controls were used for the study. Jejunum was removed from animals anaesthetised with pentobarbitone sodium (60 mg. kg^{-1} I.P.). Glucose uptake across the BBM was measured using everted sleeves, an accepted method of BBM glucose uptake using intact tissue. Addition of the SGLT1 blocker phlorizin in the uptake buffer allowed quantitation of the SGLT1 and GLUT2 components of uptake. In parallel studies, purified BBM was prepared from either fresh intestine or using tissue that had been rapidly frozen in liquid N_2 after its removal from the animal and stored at -80ºC. In both cases BBM samples were stored at -20ºC prior to their use for detection of GLUT2 and SGLT1 by western blotting. Blood glucose concentration in GK rats was higher than that in non-diabetic animals. Diabetes was without effect on SGLT1-mediated glucose uptake measured using 20 mM glucose, whereas the rate of phlorizin-insensitive (GLUT-mediated) uptake was 51.4% greater in GK jejunum (mean±SEM: 0.42±0.05 vs 0.63±0.04 nmoles/mg, $p<0.005$, Student's unpaired *t*-test). GLUT2 was readily detectable in BBM prepared from unfrozen jejunum of GK rats, but the protein was present at only minimal levels in BBM that had been immediately prepared from jejunum of non-diabetic rats or from GK jejunum that had been frozen for 2-3 days prior to BBM preparation. SGLT1 expression levels were similar in BBM prepared from intestine of fresh and stored jejunum.

Our study is the first demonstration of increased intestinal glucose uptake across the BBM in type 2 diabetes and it is likely that raised GLUT2 expression is a major contributor to upregulation of transport in this condition. The failure of previous studies to detect GLUT2 at the BBM by western blotting might be explained by the common practice of freezing whole intestine or mucosa for later preparation of BBM. The reason for the differential effect of freezing on BBM expression of GLUT2 and SGLT1 expression is unclear at the present time.

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We are grateful to Diabetes UK for support of this project.

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PC31

cAMP-dependent control of epithelial Na^+ absorption persists in the presence of an inhibitor of serum / glucocorticoid-inducible kinase 1 (SGK1)

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Epithelial Na^+ absorption can be controlled by hormones / neurotransmitters that act via cAMP / PKA (e.g. vasopressin, adrenaline) and there is evidence (e.g. Vasquez et al., 2008) that the hormone-induced activation of this pathway leads to increased activity of serum / glucocorticoid-regulated kinase 1 (SGK1). SGK1 can phosphorylate residues (Ser^{221}, Thr^{246}, Ser^{327}) within Nedd-4/2, a ubiquitin ligase that targets epithelial Na^+ channel subunits (α-, β- and γ-ENaC) for internalization / degradation (Lang et al., 2006). It has therefore been suggested that, by activating SGK1, PKA might prevent the Nedd-4/2-dependent internalization of ENaC leading to increased Na^+ transport. However, not all data support this hypothesis since others have suggested that PKA can directly phosphorylate Nedd-4/2 and thus control Na^+ absorption via a mechanism that is independent of SGK1 (Snyder *et al*., 2004). The present study therefore explores the effects of an SGK1 inhibitor (GSK65094) upon the responses to cAMP-elevating drugs in a Na^+ absorbing, human airway epithelial cells (H441).

Western analyses using phospho-specific antibodies showed that 20 min exposure to cAMP-elevating drugs increased the abundance of the Ser^{221}-, Thr^{246}- and Ser^{327}-phosphorylated forms of Nedd-4/2 (Fig. 1A). This accords well with data presented by Snyder et al. (2004) although we find that these cAMP drugs also increased the overall abundance of Nedd-4/2 itself (Fig. 1A). Analysis of surface-exposed protein showed that cAMP-elevating drugs also increased the amount of β- and γ-ENaC in the membrane (Fig 1B), whilst electometric studies revealed increased of amiloride sensitive Na^+ absorption (Fig 1C). GSK65094 (10 μM, 3 h) reduced the phosphorylation status of all three residues in Nedd-4/2 although some phosphorylation of Thr^{246} persisted in the presence of this SGK1 inhibitor (Fig. 1A). GSK65904 also reduced the membrane abundance of all three ENaC subunits (Fig 1B) and suppressed the basal rate of Na^+ transport (Fig. 1C), findings consistent with the view that SGK1 is important to the control of ENaC (Lang et al., 2006). However, cAMP clearly evoked phosphorylation of Nedd-4/2-Ser^{221}, -Ser^{327} and -Thr^{246} in GSK65904-treated cells, and these responses were accompanied by increases in the membrane abundance of α-, β- and γ-ENaC (Fig 1B), and an unambiguous stimulation of electrogenic Na^+ transport (Fig 1C).

Clear responses to cAMP therefore persist when SGK1 is inhibited indicating that the cAMP / PKA-dependent control of Na^+ absorption does not involve this protein kinase (Snyder *et al*., 2004; Mansley & Wilson, 2010).

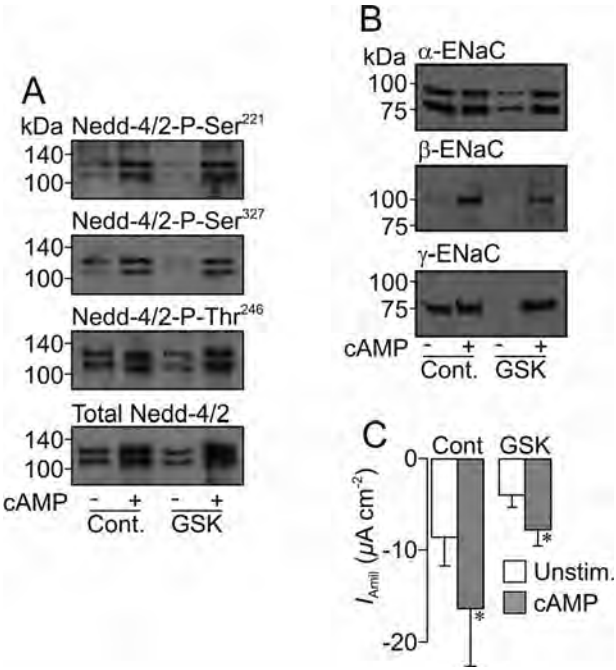

Fig. 1. Experiments were undertaken using dexamethason-treated (0.2 μM, 24 h) cells. GSK: exposed to 10 μM GSK65904 for 3 h; cAMP: exposed to 10 μM forskolin, 100 μM isobutylmethylxanthine, 1mM N6,2'-O-dibutyryl adenosine 3'5'-cyclic monophosphate for 20 min. (A) Cells were lysed in the presence of protease and phosphatase inhibitors and the Nedd-4/2 immunopurified from 1 mg aliquots of extracted protein subject to Western analysis using antibodies against the Ser^{221}-, Thr^{246}- and Ser^{327}-phosphorylated and total forms of this protein (n = 5). (B) Abundance of α-, β- and γ-ENaC in surface-exposed protein fraction isolated by biotinylation / streptavidin-binding (n = 5). (C) Amiloride-sensitive short circuit current (I_{Amil} was quantified in confluent cells mounted in Ussing chambers (n = 5; * $P < 0.05$, Student's paired t test).

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PC32

Nitrative stress increases K^+ (^{86}Rb) efflux from human placental syncytiotrophoblast

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Successful pregnancy depends on maintenance of syncytiotrophoblast (STB), the transporting epithelium of the human placenta, by cell proliferation, differentiation and apoptosis. Pre-eclampsia, a serious disease of pregnancy, is associated with abnormal STB renewal and increased nitrative stress. In many tissues cell proliferation and apoptosis is regulated by K^+ channels, including intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), and K^+ channel activity can be modulated by reactive nitrogen species (RNS). Here we test the hypothesis that STB expresses functional IK_{Ca} channels and that K^+ channels can be modulated by acute and chronic exposure to RNS.

STB K^+ channel activity was assessed by measuring ^{86}Rb (tracer for K^+) efflux from cytotrophoblast cells or villous tissue of normal term placentas (n=3-6). Cells/fragments were incubated in ^{86}Rb for 2h and then ^{86}Rb efflux measured every 1 or 2min for 10-16min. Cytotrophoblast cells were maintained in culture for 66hr until syncytia had formed. ^{86}Rb efflux was measured over 15min under control conditions and in response to acute application (over 10-15min) of peroxynitrite ($10^{-4}M$; $ONOO^-$), the IK_{Ca} channel opener EBIO (100μM) or blocker TRAM34 (10μM). To assess chronic exposure to RNS, placental tissue explants were cultured at $6\%O_2$ (normoxia for placenta at term) for 6 days. On days 3-5, explants were treated daily with EBIO (100μM) or the free radical generator SIN-1 (2.5mM) and ^{86}Rb efflux was determined at day 6. $\%^{86}Rb$ efflux was expressed as ($^{86}Rb\ efflux_t/^{86}Rb$ in cells or $tissue_o$)x100 and data were analyzed by two-way ANOVA or Wilcoxon signed rank test.

EBIO promoted an 8.4 and 1.6-fold increase in ^{86}Rb efflux from cytotrophoblast cells and freshly isolated villous fragments respectively ($p<0.04$) that was almost completely blocked (>90%) by TRAM34. $ONOO^-$ induced a sustained increase in ^{86}Rb efflux from cytotrophoblast cells (2.8x control; $p<0.0001$) and this efflux was inhibited by 5mM Ba^{2+} (20%; $p<0.01$) and TRAM34 (40%; n=2). ^{86}Rb efflux over 10min was increased by 74% and 27% ($p<0.01$) in placental explants treated with EBIO and SIN-1 respectively. EBIO and SIN-1 did not damage cell integrity as the release of lactate dehydrogenase into the culture medium was similar to control.

EBIO stimulation of ^{86}Rb efflux from STB is inhibited by TRAM34 indicating the presence of IK_{Ca} channels. Short term nitrative stress ($ONOO^-$) elevates ^{86}Rb efflux in part through activation of IK_{Ca} channels. Longer term exposure of placental villous tissue to RNS (SIN-1) promotes ^{86}Rb efflux in common with EBIO. Further experiments are needed to confirm whether IK_{Ca} channels in STB are a direct target of RNS and whether activation of these channels by nitrative stress in pre-eclampsia could contribute to altered trophoblast turnover.

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PC33

In Vivo Characterisation of Murine Tracheal Ion Transport

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Airway epithelial ion transport is responsible for maintaining airway surface liquid composition and depth, an integral defence barrier in the lung. In Cystic Fibrosis, mutations in CFTR result in aberrant Cl^- transport, resulting in airway dehydration, inflammation and infection. An alternative route for Cl^- transport is the Calcium activated Cl^- Channel (CaCC) (Ferrera *et al*., 2009). Our aim was to assess CaCC and CFTR-mediated Cl^- secretion in the murine trachea using a perfusion potential difference model. Male Balb/c mice (20-25g,Charles River, UK) were anaesthetised (16mg/mL ketamine/ 2mg/mL xylazine i.p in water for injections), secured supine and tilted nose down. Solutions (all containing 1mM amiloride) were perfused at 25μL/min onto the tracheal lumen via a custom manifold, using a Pump 22 syringe pump (Harvard Apparatus, UK). Potential difference was measured using 3.5% agar/HBSS-bridge electrodes connected to calomel half cells in 2M KCl. The exploring electrode was placed in contact with the perfusate, the reference subcutaneously in the animals flank. The manifold had a 5cm extruded tubing output, placed 5mm into a transverse incision in the distal trachea. PD was measured via a high impedance voltmeter and Powerlab (World Precision Instruments, UK). Data are expressed as absolute or Δ mean TPD ± s.e.mean (mV). All Δ values are compared to PBS (initial study only), or Cl^- free PBS baseline. For the initial study (n=9) solutions were serially perfused, determining a baseline PD (PBS Biosera, UK) of -1.5 mV ± 0.2, then establishing a Cl^- gradient (Cl^- free PBS, Biosera, UK), which induced a depolarisation of Δ +1.4mV ± 0.2. Subsequent perfusion (in Cl^- free PBS) of the CFTR blocker $CFTR_{inh}$-172 (40μM, Merck, UK) further depolarised the PD (Δ +2.2 ± 0.2mV). Further perfusion of Cl^- free PBS and $CFTR_{inh}$-172 with 10mM Carbachol (Sigma-Aldrich, UK) an M3 muscarinic agonist, induced a hyperpolarisation of Δ -3.4 ± 0.5mV. Finally, 50μM $CaCC_{inh}$-A01, a CaCC inhibitor (De La Fuente *et al*., 2008), partially inhibited the carbachol-induced hyperpolarisation (Δ +0.4 ± 0.5mV, n=9). All solutions contained 0.45% DMSO. In order to stimulate CFTR activity, (Salinas *et al*., 2004) 10μM Forskolin was perfused (in Cl^- free PBS) inducing a hyperpolarisation Δ -2.1 ± 0.4mV (n=7, 0.6% DMSO), which was partially inhibited by 40μM $CFTR_{inh}$-172 (Δ -1.5 ± 0.5mV). In a separate study, 10μM forskolin (in Cl^- free PBS, 0.6% DMSO, n=7) induced a hyperpolarisation (Δ -1.7 ± 0.3mV) which was inhibited by 100μM $CaCC_{inh}$-A01 (Δ -0.6 ± 0.3mV). The hyperpolarisation induced by carbachol, which will cause a rise in intracellular calcium, in the presence of

$CFTR_{inh}$-172, indicates that murine tracheal Cl^- transport is largely CaCC mediated. Furthermore, our results indicate that the forskolin response is mainly CaCC rather than CFTR mediated in the murine trachea.

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PC34

The contribution of BK channels to chloride efflux elicited by purinergic agonists in equine sweat gland epithelial cells

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The efflux of both chloride and potassium ions are important for secretion in equine sweat gland epithelial cells. Purinergic agonists have been shown to increase apical chloride efflux in these cells (Jenkinson et al. 2006). The mechanisms involved in purinergic-induced chloride secretion are not fully known. The efflux of chloride ions is maintained by a hyperpolarising efflux of potassium ions from the cells. Calcium-activated potassium channels (BK channels) have been identified in these cells (Huang et al. 1999), however, it is not known if these channels support chloride efflux in equine cells. The study investigated the involvement of BK channels in supporting the chloride efflux elicited by purinergic agonists.

Equine sweat gland epithelial cells were grown on permeable supports and mounted in Ussing chambers for electrophysiology. Paxilline, a potent BK channel blocker, was applied to the cells apically, basolaterally, or both simultaneously and the responses to apically applied ATP and UTP were monitored under open circuit conditions. Statistical analyses were carried out using Student's t-test, where $P<0.01$ was considered significant. Results are presented as mean ± SEM.

Blocking of BK channels, either apically or basolaterally only, had no effect on chloride efflux brought about by stimulation with ATP or UTP. When BK channels were blocked apically and basolaterally simultaneously, chloride efflux in response to ATP was not affected, however, there was a significant increase in chloride efflux in response to UTP (see figures 1 & 2).

These results indicate that the mechanisms of chloride efflux differ in response to ATP and UTP in equine sweat gland epithelial cells. BK channels do not appear to have any involvement in ATP-induced chloride efflux in these cells, whereas the UTP results suggest that BK channels have an inhibitory effect on secretion, as blocking these channels significantly increased chloride efflux from these cells.

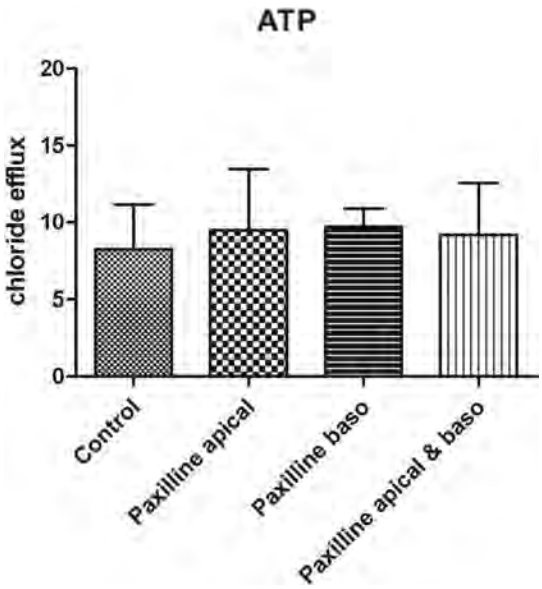

Figure 1. Increases in chloride efflux in response to ATP in the presence and absence of Paxilline.

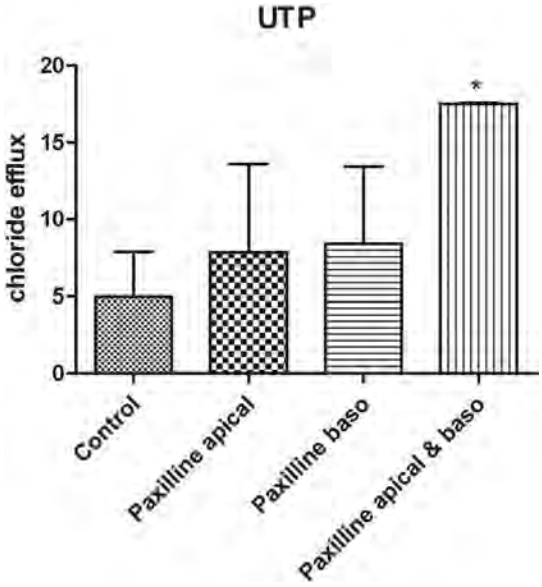

Figure 2. Increases in chloride efflux in response to UTP in the presence and absence of Paxilline. (* P<0.01)

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PC35

Restoration of channel function to the cystic fibrosis mutant A561E-CFTR by small-molecule potentiators that rescue F508del-CFTR

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Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. The commonest CF mutation F508del-CFTR has both protein processing and channel gating defects. Small-molecule potentiators have been identified which target the gating defect of F508del-CFTR (1). However, little is known about their effects on other CF mutations. Here, we studied A561E-CFTR, the second commonest CF mutation in Portugal. Like F508del, A561E is a temperature-sensitive processing mutant located in the first nucleotide-binding domain (2), which severely disrupts channel gating (wild-type (wt), open probability (P_o) ~ 0.42; A561E, P_o ~ 0.06). To rescue the A561E gating defect with CFTR potentiators, we used BHK cells stably expressing A561E-CFTR and the iodide efflux assay; BHK-wt- and F508del-CFTR were studied as controls. We tested the potentiators PG-01 (P2), SF-03 (P3), UC_{CF}-853 (P4), ΔF508act-02 (P5) and UC_{CF}-180 (P9) (all from CF Foundation Therapeutics Compound Collection, and used at 10 μM in the presence of forskolin, 10 μM); genistein (50 μM) was used as a reference potentiator. At 37 °C, forskolin and genistein elicited a large transient iodide efflux from BHK-wt-CFTR, but were without effect on BHK-A561E- and BHK-F508del-CFTR. After low-temperature incubation for 24 h, genistein partially restored channel activity to A561E- and F508del-CFTR (84 ± 5, 74 ± 3%, respectively, means ± S.E.M, n = 4), relative to the response of BHK-wt-CFTR (termed, %wt-gen). All potentiators tested enhanced iodide efflux from both BHK-A561E- and F508del-CFTR (n = 4, $P < 0.05$, unpaired T-test versus vehicle DMSO). However, none of them restored channel activity to wt-CFTR levels. For BHK-A561E-CFTR, the rank order of potentiation was P4 ≥ P9 ≥ P2 > P5 ≥ P3 (51 ± 3, 38 ± 5, 33 ± 2, 15 ± 2, 14 ± 1, respectively, %wt-gen, means ± S.E.M, n = 4) and for BHK-F508del-CFTR, it was P2 ≥ P5 ≥ P4 ≥ P3 ≥ P9 (22 ± 2, 20 ± 1, 19 ± 3, 15 ± 3, 10 ± 1, respectively, %wt-gen, means ± S.E.M, n = 4). Examination of the concentration-response relationship for P9 revealed that the half maximal effective concentration (EC_{50}) for potentiation of wt- and A561E-CFTR was similar (~ 5 μM), whereas that of F508del-CFTR was higher (~ 10 μM). In conclusion, our data suggest that all tested small molecules that

potentiate F508del-CFTR also enhance A561E-CFTR channel function, and the overall potentiating effects are better in A561E-CFTR expressing cells. However, variations in the rank order and also in P9 affinity may indicate that these two-mutant CFTRs disrupt channel gating through distinct mechanisms.

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PC36

Characterising Artificial Anion Transporters Using the Planar Lipid Bilayer Technique

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Failure of anion transport across cell membranes causes diseases, such as cystic fibrosis. One therapeutic strategy is to replace dysfunctional anion channels with alternative transport systems. Although selective cation transporters are well known, until recently anion transporters were unavailable. We previously demonstrated that cholapods, molecules derived from cholic acid, bind anions with high affinity and promote anion efflux from liposomes [1]. In the present study, we characterised the cholapods AS09, LJ09 and TL145 using the planar lipid bilayer technique. Membranes were formed using a mixture of POPE/Cholesterol (7/3) plus cholapod at a ratio of 250:1 and were bathed in symmetrical anion-containing solutions (10 – 200 mM). Membranes were voltage clamped at 0 mV and 5-second voltage steps from -200 mV to +200 mV applied to the membrane. In response, currents relaxed to a steady state after an initial peak. Using steady state currents, I-V relationships were constructed and anion conductance calculated; using current relaxations, electrical relaxation analysis was performed [2].

All three cholapods conduct Cl^-, NO_3^- and Br^-. Transport is anion concentration-dependent and anion conductance decreases with increasing anionic radius. The Cl^- and Br^- conductances of AS09 are larger than those of TL145 and LJ09, whereas TL145 has the largest NO_3^- conductance; none of the cholapods conduct SO_4^{2-}. The cholapods are highly anion selective and their anion permeability sequences are $NO_3^- > Br^- > Cl^-$.

Typically, transporter-mediated ion transport across membranes involves four steps: (i) ion and transporter form a complex on one side of the membrane, (ii) movement of the complex across membrane to the other side, (iii) dissociation of the ion from the transporter and (iv) movement of the ion-free transporter back across

membrane. The speed of these four steps is represented by four rate constants: K_R, K_{MS}, K_D and K_S, respectively.
Our analysis reveals two important points. First, values of K_{MS} of all the cholapods are smaller than those of other rate constants. This suggests that movement of anion-transporter complex across membrane is the rate-limiting step of cholapod-mediated anion transport. Second, for LJ09, values of K_{MS} and K_S are significantly smaller than those of AS09 and TL145. This provides an explanation for the small Cl^- conductance of LJ09. We conclude that to develop better anion transporters, future studies should focus on improving the lipid solubility of cholapods, their charge-shielding properties and anion affinities.

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

PC37

Rescue of F508del-CFTR chloride channel expression, gating and stability at the cell surface by the small-molecule CFTR corrector CF-106951

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F508del, the commonest cystic fibrosis (CF) mutation, causes defects in trafficking, stability and gating of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. To rescue these defects, small-molecule correctors and potentiators are being developed. Here we investigate the rescue of F508del-CFTR by the CFTR corrector CF-106951 (C18, CF Foundation Therapeutics compound collection) using inside-out membrane patches from BHK cells expressing wild-type (wt) and F508del-CFTR incubated at 37 °C. As a control, we studied low temperature corrected F508del-CFTR. The pipette (external) solution contained 10 mM Cl^- and the bath (internal) solution contained 147 mM Cl^-, 1 mM ATP and 75 nM PKA at 37 °C; voltage was -50 mV.
The gating behaviour of wt-CFTR is characterized by bursts of openings interrupted by brief closures and separated by longer closures (open probability (P_o) = 0.42 ± 0.04, mean burst duration (MBD) = 187 ± 24 ms, interburst interval (IBI) = 155 ± 19 ms, n ≥ 5). In contrast, low temperature (27 °C for 48-72 h) corrected F508del-CFTR is characterized by short bursts of openings separated by prolonged closures (P_o = 0.054 ± 0.006, MBD = 95 ± 6 ms, IBI = 1584 ± 149 ms, n ≥ 20). When pre-treated with corrector C18 (5 μM) for 24 h at 37 °C, F508del-CFTR had intermediate bursts of openings separated by shortened long closures (P_o = 0.16 ± 0.02, MBD = 140 ± 25 ms, IBI = 894 ± 108 ms, n ≥ 6, $p < 0.05$, Student's t-test). When compared with low temperature corrected F508del-CFTR, drug correction caused

a three-fold increase in P_o by prolonging MBD 47% and reducing IBI 44%. Therefore, C18 is more effective at rescuing F508del-CFTR gating than low temperature incubation.

In contrast to wt-CFTR, at 37 °C, F508del-CFTR is unstable and rapidly runs down in excised membrane patches ($t_{1/2} \approx 2$ min). We therefore investigated whether C18 might restore F508del-CFTR stability at 37 °C. Although C18 correction did not eliminate channel run-down, it delayed the complete run-down of F508del-CFTR ($t_{1/2} \approx 8$ min).

Like F508del, A561E causes trafficking and severe gating defects ($P_o = 0.06 \pm 0.01$, $n = 7$)[1]. Upon correction by C18, cell surface expression of A561E-CFTR was observed. However, C18 failed to rescue A561E-CFTR channel gating ($P_o = 0.04 \pm 0.01$, $n = 9$).

In summary, the small-molecule corrector C18 restored channel expression, partially rescued F508del-CFTR channel gating and improved stability at the cell surface. The fact that C18 restored channel gating to F508del but not A561E-CFTR suggests that the two mutations might cause trafficking and gating defects by distinct mechanisms.

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PC38

How do Na/Pi cotransporters (Slc34a) recognise phosphate?

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Phosphate (Pi) levels are tightly controlled in humans and elevated Pi in the blood may lead to atherosclerosis and endothelial dysfunction. This is a particular problem in patients with renal failure on dialysis who often suffer from hyperphosphataemia. The current strategies to reduce intestinal Pi uptake in these patients are accompanied by unwanted side effects. Pi transport in the gut is mediated by a member of the Na/Pi cotransporter family, Slc34a2 or NaPi-IIb. Detailed knowledge of how NaPi-IIb recognises and binds Pi may therefore lead to improved blockers of intestinal Pi uptake.

We have pursued a comparative approach to identify candidate regions that influence Pi affinity and specificity of NaPi-IIb. To investigate Pi affinity we have taken advantage of two NaPi-IIb isoforms from zebrafish that show very close homology on protein level but a 10-fold difference in Km for Pi. Within the functionally relevant regions of the protein (two stretches of 51 amino acids) there are 13 substi-

tutions between the two isoforms. We have performed site-directed mutagenesis and characterised the mutated transporters in Xenopus oocytes. The aim was to gradually shift the Km for Pi from one isoform towards the other.Preliminary results confirm the validity of our approach. A detailed analysis of the different mutants will be presented.

Our hypothesis predicting how Slc34a proteins recognise Pi is based on the crystal structure of a bacterial Pi binding protein. Here, a negatively charged acidic side chain stabilises the hydroxyl group of Pi. If the acidic residue is changed to a basic amino acid the protein no longer binds Pi but sulphate (SO42- in contrast to HPO42-)[1]. A sequence alignment of all known Slc34a isoforms identified two highly conserved acidic residues within the functionally important region of the transporter family. We mutated the relevant amino acids in flounder NaPi-IIb (D181 and E424) to cysteine or histidine with the aim of changing substrate specificity of the mutants from phosphate to sulphate. The mutants were expressed in Xenopus oocytes and assayed for transport of both substrates. Preliminary results revealed that the mutants E424C, D181C/424C and D181H/E424H did not transport neither Pi nor sulphate. The other mutants (D181C, D181H, E424H, D181C/E424H ans D181C/E424H) still mediated Pi induced currents. At present, sulphate transport could not be reliably measured in any of the mutants.

To conclude, our comparative approach combined with site-directed mutagenesis and functional analysis may reveal clues how Slc34a transporters interact with Pi.

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PC39

Transport of thyroxin from blood to CSF by the isolated perfused choroid plexus epithelium of the sheep: role of multi-drug resistance 1 and organic anion transporters

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Thyroxin (T_4) is essential for brain development. It is synthesized outside the central nervous system (CNS), and enters brain either directly across the blood-brain barrier or indirectly via the choroid plexus epithelium (CPE), the blood-cerebrospinal fluid (CSF) barrier. However the transport mechanism/s of T_4 across the CPE remains unclear. The current study characterised the steady-state T_4 transport from blood to CSF, across the CPE from the lateral ventricle of sheep. Sheep were anaesthetized

(20 mg.kg^{-1} thiopentone, I.V.) heparinised and exsanguinated. The brain was quickly removed and the CPE perfused via the internal choroidal arteries with albumin Ringer solution containing tracer levels of ^{125}I-T_4 (90 pM) and ^{14}C-mannitol, a non-transportable vascular permeability marker. All procedures were in accordance with Animals (Scientific Procedures) Act 1986. After 1 hr of perfusion, steady state extraction of ^{125}I-T_4 was achieved, then either 10 μM verapamil (inhibitor of MDR1 (multidrug resistance)) or 100 μM probenecid (inhibitor of Oatp2 (organic anion polypeptide)) was added to the Ringer, and ^{125}I-T_4 extraction measured for a further 45 min. In the presence of verapamil the net ^{125}I-T_4 extraction was reduced by about one third (34.3 ± 2.0%, n=10) compared to controls. Probenecid had a greater effect, reducing the extraction by almost half ($P<0.05$, student paired t-Test). We conclude that 125I-T_4 may be transported across the CPE of the sheep via Oatp2 located at the basolateral membrane and via MDR1, localized sub-apically (inner membrane of the apical surface of the CPE). However, further work is needed to determine the role of other transporters, such as Oatp1 & Oatp3, in T_4 transport across the CPE of the sheep. These transporters may play a significant role in the maintenance of T_4 concentration in CSF and brain interstitial fluid.

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PC40

Genes of the kynurenine pathway and their role in Huntington's disease

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Huntington's disease (HD) has conventionally been treated as a disease of the brain, specifically of the medium spiny neurons whose dysfunction and death is a characteristic feature of HD pathology, and which lead to the chorea associated with the disorder. However, the huntingtin protein is ubiquitously expressed, and increasingly HD is being appreciated as a whole body disorder. Indeed, one of the features of HD is the perturbation of the kynurenine pathway of tryptophan degradation, which can be detected in blood samples drawn from the periphery. In Humans, kynurenine can be broken down into both the neurotoxic free radical generator 3-hydroxykynurenine (3-HK), and the neuroprotective free radical scavenger kynurenic acid (KYNA). In HD patients the balance of this synthesis is shifted such that much more 3-HK is produced - a process we have recently shown to play a key role in HD mediated neurodegeneration (Campesan et al., 2011).
Recent work in R6/2 HD model mice found that pharmacological inhibition of the KMO enzyme responsible for 3-HK synthesis using the prodrug JM6 was significantly neuroprotective, despite the JM6 compound not crossing the blood brain barrier (Zwilling et al., 2011). In this paper, Zwilling et. al. propose a model in which the

to CSF, across the CPE from the lateral ventricle of sheep. Sheep were anaesthetized

action of JM6 leads to elevated levels of kynurenine in the blood, which is then actively transported into the brain, stimulating increased formation of neuroprotective KYNA in astrocytes.

Our research therefore seeks to augment HD therapeutics targeting the kynurenine pathway by identifying transporters responsible for the active transport of kynurenine, building on the identification LAT1 as a tryptophan/kynurenine exchanger (Kaper et al., 2007). To address this we work in Drosophila, which utilise kynurenine pathway metabolites to synthesise brown pigments called ommochromes, which are deposited as UV filters in the eyes. Mutations affecting both the synthesis and transport of kynurenine pathway metabolites therefore manifest as changes in eye colour due to changes in ommochrome levels, and to date over 85 such mutants have been found (Lloyd et al., 1998).

We have recently mapped a series of these classical eye colour mutants to the Drosophila genome sequence using standard genetic techniques, uncovering new catalytic enzymes and transporters acting in the kynurenine pathway. We are currently characterising the role of these genes in detail, with particular emphasis on their potential for augmenting the action of JM6 in the treatment of HD.

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SA01

Paracellular transport of Ca^{2+} and Mg^{2+} in the kidney

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The paracellular reabsorption of Ca^{2+} and Mg^{2+} in renal tubules plays an important role for the regulation and homeostasis of these cations. The genetic analysis of rare human disease phenotypes significantly contributed to our current understanding of the physiology of paracellular transport. In this context, the identification of mutations in CLDN16 as the underlying cause of a rare Ca^{2+} and Mg^{2+} -wasting tubular disorder was of special importance for further research activities (Simon et al, 1999). CLDN16 encodes a tight junction protein of the claudin family. Claudins have been shown to be the key components of the tight junction protein complexes which allow the formation of ion selective paracellular pathways varying from one tissue to the other. As an example, it has been demonstrated that claudin-16 and claudin-19 require each other for assembly into tight junctions in the thick ascending limb and are both parts of a cation selective paracellular "channel".
In a recent genome-wide association study, polymorphisms in CLDN14 were associated with nephrolithiasis. The exact mechanism which is responsible for this association is not known, but there might be a direct role of claudin-14 for renal Ca2+ reabsorption since CLDN11/14 double knockout mice have slightly increased urinary excretion rates for Ca^{2+} but also for Mg^{2+}. Alternatively, CLDN14 could be involved in acid-base homeostasis because it has been reported to act as a negative regulator of protons, bicarbonate and ammonium ions in the distal nephron.

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SA02

PTH-independent regulation of blood calcium concentration by the calcium-sensing receptor

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The calcium-sensing receptor controls parathyroid hormone (PTH) secretion and thereby sets blood calcium concentration. However, it is unclear whether the CaSR in other tissues can modulate blood calcium concentration in a PTH-independent manner. We investigated the role of CaSR in thyroparathyroidectomized, PTH-supplemented, and in aparathyroid rats. Chronic inhibition of CaSR selectively increased renal tubular calcium absorption and blood calcium concentration without altering bone Ca release or intestinal Ca absorption. In the kidney, CaSR was found to be mostly expressed in the thick ascending limb of the loop of Henle (TAL). CaSR inhibitors selectively increased calcium reabsorption and paracellular pathway permeability in in vitro microperfused TAL. Opposite results were obtained by chronic activation of the CaSR. We conclude that extraparathyroid CaSR is a determinant of blood calcium concentration independent of PTH, mainly by modulating renal tubular calcium transport. CaSR inhibitors may provide a new specific treatment of disorders related to impaired PTH secretion, such as hypoparathyroidism.

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SA03

EAST syndrome: a newly recognised channelopathy

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Recently, we described children presenting with a unique constellation of symptoms, namely epilepsy, ataxia, sensorineural deafness and tubulopathy, which we assigned the acronym EAST syndrome. Genetic investigations in an informative kindred with four affected children revealed the underlying genetic basis as recessive mutations in the potassium channel KCNJ10.
Affected children identified so far all presented with grand-mal seizures in infancy and were subsequently found to have ataxia and sensorineural deafness.
Biochemical investigations show a hypokalaemiac, hypochloraemic metabolic alkalosis with hypomagnesaemia and hypocalciuria, a constellation typical for impaired salt-transport in the distal convoluted tubule.
Investigation of EAST syndrome reveals the role of KCNJ10 in human physiology and pathophysiology.

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SA04

Disease mechanisms in EAST syndrome due to mutations in KCNJ10

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INTRODUCTION: Mutations in KCNJ10 lead to epilepsia, ataxia, sensorineural hearing loss and a tubulopathy, with hypomagnesemia and mild salt loss reminiscent of Gitelman's syndrome. All mutations studied so far affect channel activity and/or trafficking. To study the effect of KCNJ10 loss of function in brain, we generated a zebrafish model of the disease
OBJECTIVES: KCNJ10 mutations were expressed in mammalian cells and oocytes to investigate the properties of mutant channels in more detail. Previous data had suggested that some mutations are partially functional and we hypothesized – based on structural considerations, that these mutations impinge on channel regulation. Animal models were studied to elucidate physiological aspects of KCNJ10 function.
METHODS: KCNJ10 WT and R65P, G77R and R175Q were expressed in CHO cells and oocytes and assessed using whole cell patch-clamp, single channel recordings and macropatch recordings. Epileptic activity was recorded in morpholino knockdown zebrafish using a new recording technique.
RESULTS: All mutations investigated strongly reduced the open probability of KCNJ10 and KCNJ10/KCNJ16 heteromeric channels. R65P led to a reduction in mean channel open time, and all mutations shifted the pH-dependence of activation to more alkaline pH values. R175Q showed a strong reduction in PIP2 affinity.
Zebrafish injected with MO against KCNJ10 showed rhythmic abnormal motor patterns and hyperactivity in the optic tectum, resembling activity observed after treatment with a known seizure-inducing agent.
DISCUSSION: These results provide a framework to understand, and possibly modify, loss of function in patients with mutations in KCNJ10. In the kidney, KCNJ10 is critically involved in salt reabsorption via NCC, both by providing pump-leak coupling for the Na+/K+-ATPase as well as by providing the driving force for chloride exit through ClC-K/barttin channels. KCNJ10 is critical for stabilizing the neuronal membrane voltage by allowing glial cells to remove K+ from sites of high neuronal activity, and it is critical for the generation of the endocochlear potential. MO knockdown zebrafish are a valuable model to study the neuronal phenotype of KCNJ10 deficiency.

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SA05

ATP-sensitive potassium channels and neonatal diabetes: a treatable channelopathy

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ATP-sensitive potassium (K_{ATP}) channels are metabolic sensors that couple the metabolic state of the cell to the electrical activity of the plasma membrane. They consist of pore-forming Kir6.2 and regulatory sulphonylurea receptor (SUR) subunits. Metabolic regulation of channel activity is mediated by changes in intracellular adenine nucleotides: ATP closes the channel by binding to Kir6.2, whereas Mg-nucleotide interaction with the nucleotide-binding domains of SUR1 stimulates channel activity and reverses channel inhibition by ATP. K_{ATP} channels mediate metabolic regulation of electrical activity in numerous tissues, including neurones, cardiac and skeletal muscle and endocrine cells. In pancreatic beta-cells, K_{ATP} channels are open at rest, hyperpolarizing the cell and inhibiting insulin release. Glucose metabolism stimulates insulin secretion by closing K_{ATP} channels, depolarizing the beta-cell and opening voltage-gated Ca^{2+} channels. Sulphonylurea drugs bypass the metabolic steps, and stimulate insulin secretion directly by binding to, and closing, the K_{ATP} channel. They are used to treat type 2 diabetes. Gain-of-function mutations in the genes encoding Kir6.2 (KCNJ11) and SUR1 (ABCC8) can cause neonatal diabetes, and some mutations produce a severe clinical phenotype, characterized by developmental delay, epilepsy, muscle weakness and neonatal diabetes, Many patients have been able to switch from insulin injections to oral sulphonylurea therapy, and in some individuals the neurological symptoms can also be partially alleviated. This lecture will discuss the mechanisms by which neonatal diabetes mutations affect K_{ATP} channel function and consider how increased K_{ATP} channel activity produces the disease phenotype. Using an inducible mouse model of neonatal diabetes, we found that selective expression of gain-of-function mutations in insulin-secreting cells produced pronounced hyperglycaemia and a reduction in insulin secretion, the percentage of beta-cells/islet and insulin content, which were reversed by sulphonylurea therapy. Selective expression in neurones recapitulated the neurological problems of patients and identified novel ones.

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SA06

Genetic dissection of ENaC-mediated sodium transport along the nephron

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The Epithelial Na+ Channel (ENaC) composed of three subunits (alpha, beta and gamma) is expressed at the apical membrane of lung and renal epithelia. In the kidney, ENaC is expressed in the Aldosterone-Sensitive Distal Nephron (ASDN) where it controls sodium balance, blood volume and blood pressure. Mice with ubiquitous and constitutive gene inactivation of alpha ENaC die within 48 hours from a lung alveolar fluid clearance failure and from a severe renal salt-loosing syndrome (pseudohypoaldosteronism type 1 (PHA type 1) and thus, the consequences of total deletion of alpha ENaC in the kidney cannot be observed at adulthood. Mice in which alpha ENaC is deleted specifically in the CD are viable and do not show disturbances in sodium and potassium balance even when subjected to challenging diets (Rubera et al., JCI, 2003). This suggested that the CNT is important in controlling ENaC-mediated sodium reabsorption in the kidney. To investigate this further, we generated mice in which the alpha ENaC gene was deleted in the CD and partly in the CNT. These mice are viable until adulthood and exhibit normal BP, although they show increased urinary sodium excretion, urine output, and plasma aldosterone, leading to hyponatremia and hyperkalemia under standard diet. After sodium restriction, the mice develop a severe salt-losing phenotype and show a continuous life-threatening reduction of body weight (Christensen et al., JASN, 2010). Recently, we induced acutely a complete knockout of alpha and gamma ENaC along the whole nephron of adult animals. Thus, genetically engineered mice lacking the alpha or gamma ENaC subunit in the whole nephron have been developed using a tetracycline-inducible Cre system (Pax8-rTta/LC1; Traykova-Brauch M. et al., Nat. Med., 2008) active along the entire nephron, except the glomeruli with the floxed Scnn1a mouse (Hummler E. et al., Genesis, 2002). Upon normal salt diet and following five days of doxycycline treatment, 4 week-old alpha and gamma ENaC nephron-specific knockout mice kept loosing body weight, whereas control mice continued to gain weight normally. The XXX a KO mice develop a severe and lethal PHA-1 phenotype and are hyponatremic and severely hyperkalemic with daily cumulative loss of urinary sodium and gain of urinary potassium. Whereas, surprisingly, XXX ENaC KO mice are normonatremic but severely hyperkalemic with no significant daily cumulative loss of urinary sodium but a dramatic cumulative gain of urinary potassium. This unexpected dissociation between Na+ and K+ transport suggest that K+ secretion is no longer tightly coupled to sodium reabsorption along the entire (or some part) of the ASDN.

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SA07

Evolution of ENaC and Na,K-ATPase as limiting factors of aldosterone action

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The kidney of vertebrates plays a major role in the homeostasis of the extracellular fluid. Despite large changes in salt intake, the kidney is able to maintain the extracellular osmolarity and volume within very narrow margins. During the evolution of vertebrates, aldosterone played a critical physiological role about 300 million years ago with the emergence of amphibia, the first vertebrates to adapt to a terrestrial environment. Comparative studies of physiology, biochemistry, and molecular biology have helped in delineating the most significant steps involved. In the present study we have focused on the evolution of ENaC and Na,K-ATPase, which are the key effectors and limiting factors of the aldosterone response in the mammalian distal nephron. By searching for homologs in various eukaryotes, from unicellular eukaryotes ("protists") to multicellular metazoan, we provide here a novel view of how mammalian aldosterone-dependent control of sodium homeostasis, blood volume and blood pressure might have evolved. We propose that Na,K-ATPase emergence, together with ENaC/Degenerin, is linked to the development of multicellularity in the Metazoan kingdom. The establishment of multicellularity and the associated extracellular compartment ("internal milieu") precedes the emergence of other key elements of the aldosterone signaling pathway.

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SA08

Acid-base transport and neuronal activity

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Neuronal activity and the concomitant production of metabolic acids can vary enormously. Mechanisms for acid extrusion are therefore critical for neuronal homeostasis. Different members of the Slc4a family of bicarbonate transporters are expressed in neurons. To study their role for neuronal acitivity in more detail, we generated mouse models with a targeted disruption of the Na+ independent anion-exchanger Ae3 and the Na+ dependent anion-exchangers NCBE and NDCBE. The analysis of these mouse models unraveled important functions of these transporters both at the level of the neuronal network and synaptic transmission.

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SA10

Regulation of intestinal glucose transport by the local renin-angiotensin system

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Glucose movement across the intestinal brush border membrane (BBM) utilises the sodium-dependent transporter, SGLT1 and the facilitated transporter GLUT2. The latter is the predominant pathway when luminal glucose levels are at their peak during digestion of dietary carbohydrate. The two transport routes are linked in that SGLT1-mediated uptake is necessary for insertion of GLUT2 into the BBM in response to locally raised levels of glucose (1). Enterocyte glucose transport is a highly regulated process; adaptation can occur within a timeframe of minutes (2) and can involve events at the BBM alone or GLUT2-mediated exit across the basolateral membrane, or at both loci. Changes in uptake are a consequence of altered expression of SGLT1 and GLUT2 proteins, controlled at endocrine, luminocrine and paracrine levels. SGLT1-mediated transport may also be influenced by the BBM electrochemical gradient (2). Established systemic influences on transport include insulin, pancreatic glucagon, GIP, GLP-2, CCK and glucose. Less information is available concerning local control of glucose transport but it is known that SGLT1-dependent glucose uptake is promoted by prostaglandin E2 or epidermal growth factor in mucosal fluid, but rapidly suppressed by luminal leptin.

Experimental type 1 diabetes mellitus (T1DM) enhances SGLT1- and GLUT2-mediated glucose movement across the jejunal BBM (1,3,4). Possible mediators of this response include raised blood levels of pancreatic glucagon, decreased levels of insulin and hyperglycaemia (1,5). Acute hyperglycaemia induced by i.v. infusion of glucose also stimulates glucose uptake (6) and the speed of the response excludes control at genomic level. Since an excessive rate of glucose absorption in diabetes will exacerbate the hyperglycaemia resulting from defective peripheral glucose uptake, SGLT1 and GLUT2 are potential therapeutic targets for control of post-prandial glycaemia in diabetes. Our recent experiments suggest that angiotensin peptides might be used to target the absorptive process. Villus enterocytes express a local renin-angiotensin system (RAS) (7). Addition of angiotensin II (Ang II) to mucosal buffer markedly suppresses phlorizin-sensitive (SGLT1-mediated) glucose transport in vitro within 4 minutes via the involvement of AT1 receptor (AT1R) located at the BBM. Na^+-dependent amino acid transport is however unaffected by Ang II (7). Ang II has only a modest inhibitory action on glucose transport measured in vivo, probably because of a reduced contribution of SGLT1 to total BBM glucose transport. Interestingly, T1DM is associated with reduced enterocyte expression of angiotensin-converting enzyme (ACE) and AT1R (4) therefore down-regulation of the action of locally produced Ang II in diabetes might explain,

at least in part, enhanced glucose uptake in this condition. Another RAS product, Ang[1-7], is produced via the action of ACE2 in many tissues (e.g. heart, liver and kidney) where its acts via the Mas receptor to oppose the effects of Ang II. Our data show that Ang[1-7] is also synthesised by enterocytes and, like Ang II, it inhibits SGLT1-mediated transport whilst recognised blockers of the Mas receptor and ACE2 enhance glucose uptake. Importantly, gene and protein expression of the intestinal ACE2-Ang[1-7]-Mas receptor axis is increased during T1DM leading to higher enterocyte levels of Ang[1-7]. Treatment of T1DM or T2DM animals with Ang[1-7] reduces post-prandial glycaemia. Therefore unlike other tissues, Ang II and Ang[1-7] peptides do not have counter-regulatory actions on enterocyte glucose uptake. However, differential changes in expression of the ACE-AngII-AT1 and ACE2-Ang[1-7]-Mas axes in response to disease may produce opposing actions on glucose uptake. Thus the RAS provides multiple pathways for the control of intestinal glucose transport; the search is now on to unravel the downstream signalling elements for the actions of Ang II and Ang[1-7] on enterocyte glucose transport.

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SA11

Untangling phosphate homeostasis networks

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Inorganic phosphate homeostasis is controlled by a complex network of regulatory mechanisms. This complexity is not only the consequence of the physiological relevance of Pi, but also of the narrow threshold that separates physiological from toxic concentrations of phosphate in blood. Pi is involved in bone formation, pH control, high-energy bonds, signal transduction, membrane and nucleic acid composition, etc., but when homeostasis is lost (e.g. during chronic kidney disease), hyperphosphatemia emerges with dramatic consequences, such as Mönckeberg's sclerosis, calciphylaxis or secondary hyperparathyroidism. To avoid it, under

physiological conditions the rate of Pi ingestion should equal approximately the rate of Pi excretion.
All mechanisms that have been described to date as regulators of Pi homeostasis end up with a control of the abundance of Pi transporters in the plasma membrane, mostly of epithelial cells. These mechanisms can be either a fast (acute, within minutes-hours) or slow (chronic, days) response, and in both cases they are of either hormonal and non-hormonal nature. Acute mechanisms of control only occur in the kidney, and they involve the insertion to or retrieval (endocytosis) from the cell membrane of existing Na-coupled Pi transporters. Chronic mechanisms involve the synthesis of new Pi transporters through transcriptional or posttranscriptional mechanisms. Changes of dietary Pi as well as parathyroid hormone and phosphatonins (eg. FGF23) act both as acute and chronic regulators, while vitamin D3, thyroid hormone, insulin and glucocorticoids are only chronic regulators.
The kidney controls Pi blood concentration through changes in the rates of Pi excretion/reabsorption. Therefore, the kidney is the target for most acute and chronic regulators, which are acting on the proximal tubular epithelial cells of the nephrons. These cells express at least four different Na-coupled Pi transporters belonging to the type II (SLC34; NaPi-IIa and NaPi-IIc), and to the type III (SLC20; Pit-1 and Pit-2) families of transporters. Of these, NaPi-IIa is sufficient to handle >90% of all reabsorbed Pi in the proximal tubule; the roles of the other three transporters are unclear. With respect to changes in dietary Pi, for example, the kidney has a fundamental role of avoiding dangerous increases of Pi in blood. When a high Pi containing diet is ingested, the kidney increases Pi excretion well before the concentration of phosphate in blood is raised. In addition, the proximal tubular epithelial cells also exhibit an autonomous mechanism to sense the concentration of Pi in the lumen of the proximal tubule (or the culture media). Both mechanisms of sensing Pi concentration ensure that the control of Pi concentration in blood is maintained, independently of the source of phosphate (i.e. of dietary or internal origin, such as bone turnover).
The intestine has been classically described as an organ simply involved in Pi absorption with only modest implications in chronic control of Pi homeostasis. Recent advances, however, are uncovering new functions, including a role as sensor of Pi concentration in the diet. Evidence now suggests that the intestine senses Pi but also signals it to the kidney, to adapt the rate of Pi excretion to the Pi concentration of the meal. This role is of critical importance to avoid increases of Pi in the blood that could be the cause of undesirable intravascular calcium-phosphate precipitation. New intestinal phosphatonin candidates, such as MEPE, could be involved in the signalling process. The molecular identification of the intestinal Pi sensor is, at present, a major challenge in Pi homeostasis research.
Finally, not only the signalling viewpoint, but also the mechanisms of Pi intestinal absorption are now being elucidated. Pi intestinal absorption can be divided into transport and paracellular routes, and the proportion involved in each route most likely depends on the concentration of Pi in the lumen. The transport component seems to be the result of the activity of three Na-coupled transporters, NaPi-IIb, Pit-1 and Pit-2, and the relative involvement of each of them is also unclear. All of them are high affinity Pi transporters, with very low apparent Km values compared

to the usual Pi concentrations in lumen. Regional expression of the transporters is non-homogeneous. For example, NaPi-IIb is mostly expressed in duodenum and jejunum in rat, but this abundance pattern is altered by changes in dietary Pi content, especially during Pi deprivation.
In conclusion, our present understanding Pi homeostasis control is far from clear, and recent discoveries are increasing the complexity picture of the mechanisms involved. The coordinated interplay of Pi concentration sensing mechanisms, phosphatonins and other components need to be clarified. Similarly, the precise role of the different Pi transporters in phosphate handling and homeostasis has to be understood, especially in the case of type III transporters, Pit-1 and Pit-2. Presumably, these challenges are going to provide exciting activities and outcomes during the next years.

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SA12

Structure-function studies on epithelial phosphate transport-molecules and mechanisms

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Inorganic phosphate (P_i) is an essential nutrient involved in a variety of cellular and extra-cellular processes. The vectorial transport of anionic P_i across the lumen of epithelia is mediated by members of two genetically unrelated protein families, SLC34 and SLC20, which catalyse transport in a Na-dependent manner (1). At the lumen of renal proximal tubule epithelia, the electrogenic SCL34A1 (NaPi-IIa) and electroneutral SLC34A3 (NaPi-IIc) are the main players responsible for reabsorption of P_i from the urine. They cotransport divalent P_i, whereas the electrogenic SLC20A2 (PiT-2) cotransports monovalent P_i. In the small intestine SLC34A2 (NaPi-IIb) is at least partly responsible for dietary absorption of divalent P_i. The membrane protein abundance determines net P_i transport and their membrane targeting and retrieval is under the control of P_i levels, together with hormonal and various circulating factors. Understanding the transport mechanism at the molecular level and identifying function-specific amino acid residues is essential for defining the physiological role of these transporters and for the development of pharmaceutical agents to target them specifically. Both SLC20 and SLC34 proteins have a predicted 12 transmembrane domain topology with inverted repeat architectures. As no 3-D structural models are available for these proteins and their bacterial homologs show no similarity to transporters whose architectures are currently identified, we must rely on indirect experimental approaches to gain structure-function information. Our studies have largely focussed

on over-expression of the proteins in *Xenopus* oocytes. By performing real-time functional assays (electrophysiology and fluorometry) in combination with site-directed mutagenesis for substituted cysteine scanning accessibility (SCAM) assays, we have identified functionally important sites, established topological features and have begun to elucidate the dynamics of protein conformation changes during the transport cycle, under physiological conditions. Using SCAM, in which novel cysteines are substituted at sites predicted to be functionally sensitive, labelled with methanethiosulfonate reagents and their accessibility from the aqueous milieu quantified, we could confirm and refine topological predictions obtained from bioinformatics. For example, the intra- and extracellular accessibility of two short reentrant regions, previously hypothesised to form the transport pathway of NaPi-IIa/b, was established. Recently, using a crosslinking strategy, we obtained compelling evidence that for NaPi-IIb, these regions physically associate (2). The marked difference in electrogenic activity for the two renal SLC34 isoforms, which otherwise show >90% sequence identity in the putative transmembrane spanning regions, prompted us to investigate the underlying molecular determinants. By means of sequence comparison, we identified and experimentally confirmed the location of critical residues that confer electrogenicity to NaPi-IIa/b and moreover, define their transport stoichiometries ($3Na^+$:P_i for NaPi-IIa /b and $2Na^+$:P_i for NaPi-IIc) and P_i concentrating capacity (3). Furthermore, we established experimentally that 3 Na^+ ions interact with the SLC34 proteins, but for the electroneutral NaPi-IIc, the first Na^+ ion to bind is not cotransported, thereby providing evidence that transport and binding stoichiometries are not necessarily equivalent (4). Recently we have applied voltage clamp fluorometry (VCF) to investigate conformational changes during the transport cycle. Novel cysteine residues at functionally important sites were labelled with fluorophores and real-time changes in fluorescence emission intensity induced by changes in membrane potential or substrate activity were detected. These indicate an altered microenvironment of the fluorophore that is sensitive to the protein conformation. Using VCF, we have established the order of cation interaction (4,5) and we have obtained evidence of complementary movements of the protein when substrates bind and debind (6). Taken together, these and other studies have allowed us to generate a kinetic model for SLC34 proteins, which provides mechanistic insight into the transport cycle.

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SA13

Intestinal phosphate transport, the renal-gastrointestinal axis, and a potential role for 'phosphatonins'

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Phosphate homeostasis is achieved through a balance between intestinal absorption and renal excretion of phosphate, as well as an internal contribution from bone. However, compared with the kidney, much less is known about the mechanisms involved in intestinal phosphate absorption or how this process may be regulated. Yet there is growing recognition of the importance of the gut in phosphate balance, especially in chronic kidney disease with progressive renal failure, a setting in which there is disturbed phosphate balance, leading to phosphate retention, overload, and serious complications such as uncontrolled parathyroid gland overactivity (secondary hyperparathyroidism) and accelerated vascular calcification with associated high cardiovascular morbidity and mortality (20% mortality over 5 years).

Classically, the type II sodium phosphate co-transporter NaPi-IIb has been considered to be the rate limiting step for intestinal phosphate absorption. However, recent studies suggest that PiT1 and PiT2 proteins, members of the SLC20 family, are also expressed at the enterocyte brush border membrane, where they may play a role in intestinal phosphate absorption, particularly during dietary phosphate restriction. The role of phosphatonins (novel phosphaturic factors such as FGF-23 and MEPE) in the regulation of phosphate balance, particularly their influence on renal phosphate handling, has been studied in some detail; although evidence is now emerging that the phosphatonins can also regulate intestinal phosphate absorption. This presentation will summarise our current understanding of intestinal phosphate transport and its regulation, its relationship to renal phosphate handling, and try to highlight the potential importance of the gut as a target in the control of hyperphosphatemia in, for example, renal failure.

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SA14

Ion transport processes in distal renal tubular acidosis (dRTA)

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This is the classical RTA disease, Type 1, not Types 2-4 which are heterogenous, rarer and less well understood. Basic defect in dRTA is renal inability of excrete urine more acid than pH 5.3 (H^+ 5 μmol/l), a fault in the alpha-intercalated cell of renal cortical collecting duct, where 3 metabolic processes are involved - 1) hydration of dissolved carbon dioxide to proton and bicarbonate, catalyzed by CA II, 2) proton secretion through apical membrane, 3) bicarbonate reclamation by anion-exchanger in basolateral membrane. dRTA can result from damage to any of these. Commonest cause of dRTA is autoimmune in post-pubertal women, causing loss of 2), 3) or both, or global destruction of whole alpha-intercalated cell. Defects in apical proton secretion from gene mutations cause occidental recessive dRTA. Defects in baso-lateral chloride/bicarbonate exchanger, through SLC4A1 mutations, cause rare but universally distributed dominant dRTA. Southeast Asian dRTA is recessive and has high local incidence, almost invariably the result of homozygous or compound heterozygous SLC4A1 mutations that have never been recorded in the west, and cause profound red-cell morphological changes and increased haemolysis. These mutations may have evolved as protection against Plasmodium falciparum malaria.

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SA15

Transport phenomena in the cochlear epithelia and their role in hearing

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The mammalian cochlear partition uses transport proteins to implement a structure which is able to encode sound frequencies in the acoustic range, that is, up to 100kHz in some species. The design principle is the use of the high mechanical resonant frequencies of small structures in order to respond to these frequencies normally beyond the range of most biological processes. The complicating factor is that the coding epithelium, the organ of Corti, operates in a fluid environment and to compensate for the dissipation due to the viscous forces several specialised processes have co-evolved.
The mammalian cochlea is a three compartment tube coiled within the temporal bone, in total length anything from 7mm in the mouse to nearly 60 mm in a whale.

One dividing partition is formed by the basilar membrane which acts as the mechanical substrate for frequency coding. The central cochlear compartment, scala media, contains the hair cells of the organ of Corti whose sensory processes project into a solution containing ca 140 mM K^+ and 30 μM Ca^{2+} as well as elevated levels of bicarbonate. Scala media is bounded on one side by a transport epithelium, the stria vascularis (SV), whose mechanisms are less well understood than they deserve. SV maintains a high (+80 to +120mV) endolymphatic potential (EP) by electrogenically transporting K^+ the basal side of the cells forming the margin of scala media. The energetic cost of maintaining endolymph is reflected in the developmental sequence that builds the compartment and the transporters involved in recirculating K+ back to endolymph. Mutations in the main K+ feed route from SV to endolymph, via the K^+ channels KCNQ1 and KCNJ10 both lead to hearing loss. Mutations in Cx26 and KCC4 in the recirculation pathway are also prime causes of deafness both in humans and in mouse models. Finally mutations in pendrin (SLC26A4), located in scala media also lead to clinically significant hearing loss but pendrin's precise role in endolymph homeostasis remains obscure (Zdebik et al, 2009).

What is the function of high positive endolymphatic potential in the mammalian cochlea? One answer that has recently emerged depends on knowing how outer hair cells (OHCs) function. These cells act as sensory cells in the organ of Corti but can also generate forces at acoustic frequencies and can compensate for cochlear viscous dissipation. The OHC force generating mechanism depends on a dense packing of prestin (SLC26A5) in the basolateral membrane. In this role, there is evidence that prestin has evolved away from its anion-bicarbonate exchange capacity in non-mammals to exhibit primarily a mechanoenzyme function in OHCs. Prestin undergoes conformational switches as a result of OHC membrane potential changes. The structural basis for the mechanism is unresolved. For the OHC voltage signal to respond rapidly enough the membrane time constant is reduced by a standing depolarizing current flowing from scala media through the apical transducer channels to activate K channels (predominantly KCNQ4) in the basolateral cell membrane (Johnson et al, 2011). The high cochlear EP appears to ensure that the OHC receptor potential is sufficient to allow prestin to track and to amplify the sound wave on a cycle-by-cycle basis.

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SA16

Dissecting a regulatory calcium binding site of CLC-K kidney chloride channels

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CLC proteins form a family of voltage-gated Cl-channels and Cl-/H+-exchangers that are found in all phyla. The human Cl- channels ClC-Ka and ClC-Kb, as their correspondent murine orthologues ClC-K1 and ClC-K2, are almost exclusively expressed in kidney and inner ear epithelia, where they are involved in NaCl reabsorption and endolymph production, respectively. CLC-K channels co-assemble with the β-subunit barttin. Mutations in ClC-Kb and barttin cause Bartter's syndrome with hypokalemia and salt wasting. Previous reports described CLC-K modulation by extracellular calcium and protons.

Currents increase with increasing [Ca2+]ext and are blocked by increasing [H+]ext. An extensive mutagenic screen, based on the crystal structure of the bacterial homologue Ec-CLC-1, combined with voltage clamp measurements on CLC-Ka led us to identify the residue responsible for proton induced block (H497) and two acidic residues responsible for Ca2+ sensitivity (E261 and D278). E261 of one subunit and D278 of the neighboring subunit are close to each other and likely form an intersubunit Ca2+-binding site. All three residues are relatively close, and thus we have identified a novel region involved in the regulation of gating of a CLC channel.

To investigate the specificity of the Ca2+ binding site we studied the effect of various divalent cations (Zn2+, Mg2+, Ba2+, Sr2+, Mn2+) on CLC-Ka, CLC-K1, and CLC-Kb. Both WT CLC-Ka and the double mutant E261Q/D278N, were blocked by 5 mM Zn2+ suggesting that Zn2+ affects the channel by binding to a separate binding site. Mg2+ does not activate CLC-Ks at concentrations up to 50 mM. In contrast, CLC-Ka was activated by Ba2+, Sr2+, and Mn2+. The rank order of potency was Ca2+>Ba2+>Sr2+=Mn2+, likely corresponding to a decreasing affinity of these cations. Furthermore, the Ca2+ insensitive double mutant, E261/D278, was also insensitive to Ba2+ and Sr2+ demonstrating the specificity of the mechanism of activation of CLC-K channels by Ca2+. The Ca2+ binding site is conserved in CLC-Kb and CLC-K1 but, interestingly, CLC-K1 showed an altered rank order Ca2+>Sr2+>>Ba>Mg2+. These results will be helpful in the development of a structural model of the cation binding site.

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SA17

Biochemical Analysis of cardiac ATP-Sensitive potassium (K_{ATP}) channels

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KATP channels were originally discovered in cardiac myocytes. Since then they have been molecularly defined, e.g. it has been shown that they are comprised of four Kir6.1 or Kir6.2 and four SUR1 or SUR2A/B subunits. Substantial progress has elucidated their role in the physiology and pathophysiology of pancreatic beta cells and cardiac myocytes. However, the biochemical description of K_{ATP} channel complexes from native cells lags behind the many functional and physiological insights that the field has generated. Here we provide a detailed characterization of Kir6.2 and SUR1 proteins in different cell types of the heart. We employ blue native gel electrophoresis, glycan analysis, affinity purification and indirect immunofluorescence to demonstrate different species of K_{ATP} channels. Our results will be integrated with current knowledge on the trafficking and cell surface expression of heterologously expressed K_{ATP} channels.

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SA18

Voltage-gated calcium channel α2δ subunits: trafficking and function

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The α2δ auxiliary subunits of voltage-gated calcium channels enhance calcium currents and affect their properties, but their mechanism of action is not well understood. We have recently shown that α2δ subunits can form glycosyl phophatidyl inositol (GPI)-achored proteins (Davies et al., 2010), and this is essential for their function, and explains their localization in lipid raft fractions (Davies et al, 2006). The anti-epileptic and anti-nociceptive drugs gabapentin (GBP) and pregabalin (PGB) are known to bind to α2δ-1 and α2δ-2, and the α2δ-1 target is essential for the antihyperalgesic action of this drug (Field et al., 2006). We have found that acute application of GBP does not affect calcium currents in several different systems. However, chronic application of GBP to cultured cells reduces both calcium currents and cell-surface expression of heterologously expressed α2δ and α1 subunits (Hendrich et al., 2008), and PGB also affects α2δ trafficking in vivo (Bauer et al., 2009). This process involves an inhibition of trafficking through the recycling

endosomes (Tran-Van-Minh and Dolphin, 2010). Our evidence indicates that gabapentinoid drugs act chronically to impair the trafficking function of $\alpha 2\delta$ subunits.

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Tran-Van-Minh and Dolphin, 2010, J Neurosci 30: 12856 –12867

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