PC3

Identification, localization and functional study of epithelial Ca^{2+} channel TRPV6 in the rat epididymis

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Calcium is known to play a crucial role in sperm physiology, including motility, metabolism, acrosome reaction, and fertilization. However, very little is known about the regulation of Ca^{2+} in the epididymis, although it is known that the fluid therein has a lower Ca^{2+} concentration than in the blood plasma. The calcium permeable channel TRPV6, but not TRPV5, is expressed in the reproductive tract of male rats as analyzed by RT-PCR and immunohistochemistry. TRPV6 is predominantly present in the apical membranes of the principal cells of the epididymis and the ciliated cells of the efferent duct. Whole-cell patch-clamp studies of isolated epithelial cells from the rat epididymis revealed a Ca^{2+}-selective current with characteristics matching those of the epithelial Ca^{2+} channels, viz constitutive activities, time-dependent inactivation at hyperpolarizing steps of membrane potentials to more negative than –60 mV, inwardly rectifying current-voltage relationship, inhibition by extracellular acidosis but stimulation by alkalosis, and blockade by lanthanum. When the cauda epididymal tubules of anaesthetized rats (pentobarbitone sodium, 60mg kg^{-1}, i.p. injection) were luminally perfused with HCO_3^--buffered Krebs solution (pH_o 7.4) *in vivo*, the perfused segment reabsorbed Ca^{2+} at a rate of 2.6 ± 0.1 nmol cm^{-2} min^{-1} (n=58 from 16 rats). Reabsorption was dose-dependently suppressed by ruthenium red and lanthanum, putative blockers of epithelial Ca^{2+} channels. Castration markedly reduced the Ca^{2+} reabsorptive capacity of the epididymal tubule (n = 16 rats). This study suggests that TRPV6 provides a Ca^{2+} entry pathway that regulates Ca^{2+} homeostasis in the epididymis. Reabsorption of Ca^{2+} by the epididymal tubule is androgen-dependent.

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

PC4

Possible role of apical P2 receptors in modulating aquaporin-2-mediated water reabsorption in the collecting duct

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Activation of basolateral V_2 receptors by vasopressin increases water reabsorption in the collecting duct (CD) via a series of intracellular events that culminate in increased insertion of aquaporin-2 water channels (AQP2) into the apical membrane. Recent studies have shown that this process is inhibited by extracellular ATP acting on P2 receptors expressed on the basolateral membrane of CD principal cells, and that $P2Y_2$-like receptors are responsible (Unwin *et al.* 2003). The present study has used the *Xenopus* oocyte expression system to investigate whether other P2 receptor subtypes can influence AQP2 function, and has localised P2 receptor distribution in the rat CD.

Defolliculated *Xenopus* oocytes (co-expressing AQP2 and a given P2 receptor) were placed in a hypotonic (10 mosm/l) Barth's solution, and AQP2-mediated swelling was monitored by video imaging at 1.7 s intervals for 1 min; from this, osmotic water permeability (P_f) was calculated (Deen *et al.* 1994). When $P2X_2$, $P2Y_2$ or $P2Y_4$ receptors were co-expressed with AQP2, then activated by 10 μM ATP, P_f was reduced significantly (by $46 \pm 8\%$ ($P<0.01$, unpaired *t* test), $53 \pm 7\%$ ($P<0.01$) and $57 \pm 3\%$ ($P<0.01$), respectively; means ± SEM; $n=8$ in each case). Western blot analysis, with a specific AQP2 antibody, confirmed that the P2 receptor-mediated inhibition of cell swelling was due to removal of AQP2 protein from the plasma membrane in each case ($n=3$).

Kidneys from three terminally anaesthetised adult Sprague Dawley rats were perfusion-fixed with paraformaldehyde (4%) and sliced (8 μm). Using antibodies specific for $P2X_2$, $P2Y_2$ and $P2Y_4$ receptors, immunohistochemical analysis demonstrated the expression of $P2X_2$ and $P2Y_4$ receptors on the apical membrane throughout the CD and $P2Y_2$ on the basolateral membrane in the inner medullary CD.

These results show directly that activation of certain P2 receptors can inhibit AQP2-mediated water transport. They also suggest that, in addition to the previously documented effect of basolateral $P2Y_2$ receptors, apically located P2 receptors ($P2X_2$ or $P2Y_4$) might play a role in modulating vasopressin-stimulated water reabsorption in the CD, thus strengthening suggestions that intraluminal nucleotides might act as paracrine/autocrine agents.

Unwin RJ *et al.* (2003). *News Physiol Sci* 18, 237-241.

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PC5

Acute regulation of the urea transporter UT-A3, expressed in a MDCK cell line

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Renal facilitative urea transporters play a vital role in the urinary concentrating mechanism (Fenton *et al.* 2004). UT-A3 is a phloretin-sensitive urea transporter expressed on the basolateral membrane of inner medullary collecting duct cells (Stewart *et al.* 2004). In this study, we have produced a MDCK cell line that stably expresses myc-tagged UT-A3, and investigated the resulting urea transport in these MDCK:UT-A3 cells. Radioactive ^{14}C-

labelled urea flux experiments showed that during basal conditions there was no difference in basolateral urea uptake into control MDCK cells (1.72 ± 0.22 nmol cm^{-2} min^{-1}, n=16) and MDCK:UT-A3 cells (1.99 ± 0.24 nmol cm^{-2} min^{-1}, n=16) (NS, unpaired t test). However, while pre-incubation for 60 min in 10^{-6} M arginine vasopressin (AVP) had no effect on urea uptake into control MDCK cells (1.94 ± 0.09 nmol cm^{-2} min^{-1}, n=4, NS, ANOVA), it significantly stimulated urea uptake into MDCK:UT-A3 cells (6.12 ± 0.68 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA); as did 60 min pre-incubation with 10^{-6} M AVP (5.23 ± 0.66 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA). Further investigation showed that this 10^{-6} M AVP response was in fact biphasic, with an initial peak after 10 min (5.31 ± 0.70 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA) followed by a larger response after 60 min (7.07 ± 0.74 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA). Importantly, the 60 min AVP response was significantly inhibited by 500 μM phloretin (73 ± 8% of control, n=4, $P<0.05$, ANOVA). Finally, MDCK:UT-A3 urea uptake was also stimulated by 10 μM forskolin (5.53 ± 0.78 nmol cm^{-2} min^{-1}, n=4, $P<0.01$, ANOVA), 250 μM 8-bromo-cAMP (3.74 ± 0.23 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA) or 1 mM ATP (4.73 ± 0.56 nmol cm^{-2} min^{-1}, n=4, $P<0.05$, ANOVA). In conclusion, our results indicate that phloretin-sensitive UT-A3 urea transport can be regulated by AVP, possibly via intracellular increases of cAMP and/or calcium.

Fenton RA *et al.* (2004). *Proc Natl Acad Sci* **101**, 7469-7474.

Stewart GS *et al.* (2004). *Am J Physiol Renal* **286**, F979-F987.

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PC6

A new role for a famous gene: *white* encodes a *Drosphila* renal cyclic GMP transporter

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Drosophila Malpighian (renal) tubules are a powerful and adaptable model for studying *in vitro* epithelial transport mechanisms. Mutations in the *white* gene were the very first identified by Morgan. *White* is one of the most important genetic markers in modern *Drosophila* molecular biology; however, its function is surprisingly poorly understood. Previous studies have shown that *white* gene encodes a member of the G family [1] of ATP binding cassette (ABC) transporters, and is implicated in the transport of kynurenine, tryptophan and guanine. However, the function of *white* is surprisingly poorly understood considering it is one of the most important markers in modern *Drosophila* molecular biology. Cyclic guanosine 3',5'-cyclic monophosphate (cGMP) is a signalling molecule involved in the regulation of a diverse range of tissues [2]. Cyclic GMP can be actively transported by various ABC transporters [3].

In a recent Affymetrix microarray experiment *white* was found to be 10.3 ± 1.4 (mean ± SEM, N=5) up-regulated in the Malpighian tubules [4] and differentially expressed when the tubules were incubated with cGMP. As the tubules are stimulated by exogenous cGMP, and are known to transport cyclic nucleotides, we hypothesised that *white* might be involved in cGMP transport. Using ^3H labelled cGMP in a transport assay [5], wild-type tubules were shown to transport cGMP at a rate of 2.22 ± 0.24 fmol min^{-1} (mean ± SEM, N=19), while in *white* mutant the rate of transport was 0.86 ± 0.09 fmol min^{-1} (mean ± SEM, N=18), significantly ($P<0.001$) less cGMP than wild type tubules. A number of pharmaceutical competitors effect on cGMP transport were characterized, including glibenclamide, methotrexate, kynurenine, and tryptophan. Interestingly cAMP does not compete with cGMP transport, suggesting that the transport of these cyclic nucleotides is independent in *Drosophila* renal tubules. The *white* protein appears to be expressed at the basolateral membrane, using ICCs and a GFP-labelled overexpression construct. Further secretion assays demonstrate that even in the presence of cGMP transport inhibitors, or in *white* mutants, cGMP stimulates fluid secretion by tubules, suggesting an independent receptor-mediated action of cGMP, that does not require transepithelial transport.

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PC7

Immunohistochemical localisation of P2 receptors in the rat renal collecting duct: effects of altering dietary sodium intake

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ATP can be detected in urine at concentrations sufficient to activate P2 receptors (P2Rs) expressed in the luminal membrane of renal tubular cells (Unwin *et al.* 2003). The activation of certain apical P2Rs by extracellular ATP has been shown to inhibit Na^+ transport in the collecting duct (CD) *in vitro* and *in vivo*. Previously, using the *Xenopus* oocyte expression system, we have demonstrated a regulatory interdependence between certain P2Rs and the epithelial Na^+ channel (ENaC) (Wildman *et al.* 2005). We have shown that plasma membrane expression of P2R assemblies incorporating $P2X_2$, $P2X_5$ and $P2X_6$ subunits are directly increased by ENaC expression and that ENaC activity is decreased by the stimulation of P2R assemblies incorporating $P2X_2$, $P2X_4$ and $P2X_6$ subunits. We have proposed that some P2Rs may provide localised and fine regulation of ENaC activity in the CD *in vivo*.

Kidneys from terminally anaesthetised adult Sprague Dawley rats (maintained on low (0.01%), normal (0.5%) or high (4%)

sodium diets, for 10 days) were perfusion-fixed with paraformaldehyde (4%) and sliced (8 μm). We used co-immunofluorescence (using an AQP2 antibody as a marker of the CD) to investigate apical P2R expression patterns along the CD in response to changes in dietary sodium intake (that also change ENaC expression).

In rats maintained on a normal Na^+ diet (n=3), immunostaining for $P2X_4$ and $P2X_6$ ion channel receptor subunits and $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ metabotropic receptors was evident in the apical membrane throughout the CD. Weak staining was seen for apical $P2X_2$, $P2X_5$ and $P2X_7$ receptors. In rats on a low Na^+ diet (n=3), expression of $P2X_1$ also became apparent, expression of $P2X_2$ was increased and immunostaining for $P2X_5$ disappeared. With the exception of $P2Y_4$, all previously detected P2Y receptors showed restricted co-localisation and weak apical staining. A high Na^+ diet also resulted in increased apical expression of $P2X_1$, but in contrast to normal and low Na^+ diets expression of apical $P2X_7$ receptors was increased and immunostaining for $P2X_2$ disappeared. We also saw weak staining for those P2Y receptors previously detected in abundance, with exception of $P2Y_6$ and $P2Y_{12}$, which remained unchanged.

In summary, apical P2R expression patterns change in the rat CD in response to changes in dietary sodium intake. Our results support a link between P2R expression and function and ENaC regulation (i.e. assemblies of $P2X_2$, $P2X_4$ and $P2X_6$ subunits, having the ability to inhibit ENaC activity, shown here to mirror ENaC expression *in vivo*), which, itself, is regulated by levels of dietary Na^+.

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E.C. and S.H. contributed equally to this work.

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PC8

A Ca^{2+}-activated Cl^- conductance supports the regulatory volume decrease of mouse renal collecting duct cells in primary culture

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Cells of the renal inner medullary collecting duct (IMCD) are able to regulate their volume after hypoosmotic perturbations (regulatory volume decrease, RVD) by activating a swelling-sensitive anion/organic osmolyte channel (VRAC) [1]. As full activation of VRAC takes several minutes it might be of advantage for the IMCD cells to recruit other anion conductances for support, especially in case of massive hypoosmotic stress. A possible candidate might be the Ca^{2+}-activated Cl^- conductance (CaCC), as it can be recruited rapidly by even a small increase in intracellular calcium ($[Ca^{2+}]_{in}$) [2].

To investigate this hypothesis, cells isolated from the initial third of the mouse IMCD (mIMCD; see [2]) were cultured on glass coverslips (Media osmolality: 600 mosmol/kg H_2O) and challenged with either a moderate (bath osmolality reduction by 100 mosmol/kg H_2O via removal of sucrose) or a massive (bath osmolality reduction by 300 mosmol/kg H_2O via removal of sucrose) hypoosmotic shock, respectively. Membrane conductance changes of the cells were investigated using the slow whole cell patch-clamp technique (Nystatin perforated patch). Currents were attributed to VRAC or CaCC by their distinct biophysical characteristics (see [1]). Alterations in $[Ca^{2+}]_{in}$ were measured by intracellular ratiometric Ca^{2+} imaging using the fluorescent calcium indicator Fura2. Data are given as mean±SEM (n), statistical significance was tested as in [1].

A moderate hypoosmotic challenge via a reduction of the extracellular osmolality by 100 mosmol/kg H_2O resulted in activation of VRAC. However, neither a significant increase in $[Ca^{2+}]_{in}$ nor an activation of CaCC was detectable.

On the other hand, challenging the mIMCD cells with a massive hypoosmotic shock (-300 mosmol/kg H_2O) elicited not only VRAC activity but also an increase in $[Ca^{2+}]_{in}$ in conjunction with activation of CaCC. $[Ca^{2+}]_{in}$ rose from a basal level of 28±5nM (n=7) to 367±21nM (n=7), and membrane conductance grew from -2.4±0.8pA/pF to -12.2±1.2pA/pF at -80mV and from 4.2±0.9pA/pF to 72±2.1pA/pF at +80mV (n=11), respectively. The increase in $[Ca^{2+}]_{in}$ could be detected 31±5s (n=10) after the start of the hypoosmotic challenge and lasted as long as the osmotic gradient was maintained.

Omission of calcium in the extracellular bathing solution ($[Ca^{2+}]_{ex} < 10$nM) had neither a significant effect on the intracellular Ca^{2+} signal nor on the CaCC activity elicited by the hypoosmotic challenge. Chelation of intracellular Ca^{2+} on the other hand prevented the $[Ca^{2+}]$in rise as well as CaCC activation. This indicates intracellular calcium stores as the main Ca^{2+} source for the signal.

In conclusion, activation of CaCC via an intracellular calcium signal seems to support the RVD of mIMCD cells after a strong hypoosmotic challenge by providing an early anion efflux pathway which precedes the slower activating VRAC conductance.

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PC9

17β-Oestradiol rapidly activates intracellular calcium signalling via a protein kinase C—protein kinase A-dependent pathway in the human eccrine sweat gland cell line NCL-SG3

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β-Adrenergic-stimulated sweat secretion is mediated by calcium and cAMP. We describe here, for the first time, protein kinase A- (PKA) and protein kinase C- (PKC) dependent modulation of intracellular calcium ($[Ca^{2+}]_i$) by the hormone 17 β-oestra-

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PC20

Properties of Ba^{2+} block of an inwardly rectifying K^+ conductance in cultured mouse collecting duct cells

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The renal collecting duct is composed of principal and intercalated cells, which serve to control the extracellular fluid volume and solute composition of the body. Inwardly rectifying K^+ channels play an important role in collecting duct function. The aim of the following study was to examine an inwardly rectifying K^+ conductance in a cultured mouse collecting duct cell line (M8). K^+ currents were examined using the whole cell patch clamp technique. Clamp potential was stepped from a holding value of –40 mV, to between +100 and -100 mV in 20 mV steps. The current sensitive to 5 mM Ba^{2+} was measured in the presence of 135 mM KCl or 130 mM NaCl plus 5 mM KCl in the bath (both at pH 7.4 with 2 mM $CaCl_2$). The pipette solution contained 145 mM KCl with no added Ca^{2+} plus 0.5 mM EGTA (pH 7.4). Measurements were taken initially (~50 mS) after changing the potential and at steady state (SS) (~350 mS). Point conductance was calculated at +100 (G_{out}) and –100 mV (G_{in}). All values are expressed as means ± SEM. Statistical significance was tested using Student's unpaired t test and assumed at the 5% level.

With high KCl in the bathing solution the initial Ba^{2+}-sensitive G_{out} was 86.58 ± 32.37 μS/cm^2 and the initial G_{in} was 159.22 ± 49.92 μS/cm^2 (n=8). G_{in} was significantly greater than G_{out} identifying a weak inwardly rectifying K^+ conductance. SS conductances were not significantly different to initial values. With high NaCl in the bathing Ringer solution the initial Ba^{2+}-sensitive G_{out} was 140.60 ± 19.81 μS/cm^2 (n=48). At 350 ms Ba^{2+}-sensitive G_{out} had fallen to –9.69 ± 4.50 μS/cm^2. The initial Ba^{2+}-sensitive G_{in} was 53.51 ± 5.45 μS/cm^2 (n=48) and this increased at 350 ms to 64.73 ± 7.76 μS/cm^2. Time-dependent changes in G_{out} and G_{in} were not observed with total whole cell conductances.

These data are consistent with time-dependent changes in Ba^{2+} sensitivity of the whole cell K^+ conductance in the presence of extracellular Na^+. One explanation is that is at positive potentials Ba^{2+} ions are repelled out of the channel pore. The absence of time-dependent changes in Ba^{2+} sensitivity in the presence of extracellular K^+ could reflect the presence of a K^+ lock-in site, which prevents Ba^{2+} from leaving the pore when K^+ is bound (Spassova & Lu 1999; Vergara *et al.* 1999).

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PC21

Basolateral K^+ conductance is decreased during cAMP-stimulated Cl^- secretion in human colonic crypts

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The current model of Cl^- secretion in mammalian colonic crypts involves electroneutral Cl^- entry across the basolateral membrane, facilitated by Na^+-K^+-2Cl^- co-transport, followed by electrogenic Cl^- exit across the apical membrane through cAMP activated Cl^- channels. Simultaneous activation of basolateral K^+ channels is thought to maintain a favourable electrical gradient for apical Cl^- secretion by limiting cell depolarisation. In this study we have sought evidence for K^+ channel activation by cAMP using perforated whole cell patch clamp in human colonic crypts.

Sigmoid biopsies were obtained from patients undergoing routine colonoscopy for altered bowel habit. Colonic crypts were isolated using a Ca^{2+} chelation technique as described previously (Bowley *et al.*, 2003). Slow-whole cell currents (with amphotericin B in the pipette) were measured from colonocytes in the mid-third of intact crypts. Single channel currents were measured from the basolateral membrane using the cell attached patch technique. Data are presented as mean ± 1 SEM, with n, the number of experiments. Comparison was by paired t test with significance assumed at $p < 0.05$.

Under basal conditions, whole cell currents were predominantly K^+ selective with a conductance (G) of 0.98 ± 0.1 nS and a reversal potential (E_{rev}) of -62 ± 2 mV (n = 40). Forskolin (FSK, 10 μM) gave a ~3 fold increase in G and a depolarising shift in E_{rev} of 29.8 ± 7 mV (n = 8, $P < 0.05$). The FSK-stimulated conductance was significantly inhibited by removal of bath Cl^- (107 ± 3%, n = 8), or addition of NPPB (200 μM; 85 ± 18%, n = 4) but not DIDS (100 μM; 1 ± 8%, n = 4), indicating activation of CFTR. The effect of FSK on GK^+ was investigated under low Cl^- conditions (4mM Cl^- in bath and pipette), where FSK reduced the basal conductance from 1.43 ± 0.6 nS to 0.56 ± 0.1 nS (n = 6, $P < 0.05$) and depolarised the cell from -62 ± 4 to -44 ± 8 mV ($P < 0.05$), consistent with K^+ channel inhibition. Addition of chromanol 293B (10 μM), an inhibitor of the cAMP-activated K^+ channel KCNQ1, to FSK-stimulated crypts, was without effect in both high and low Cl^- experiments. Single channel analysis of the basolateral membrane revealed that intermediate conductance Ca^{2+}-sensitive K^+ channels (IK_{Ca}) were inhibited by forskolin (n = 7, $p<0.05$).

In conclusion, FSK stimulated a Cl^- conductance with pharmacological properties consistent with CFTR. We found no evidence of K^+ channel activation by cAMP in human colonic crypts. Paradoxically, FSK inhibited the basal whole cell K^+ conductance and basolateral IK_{Ca} channels suggesting that basolateral K^+ channels have a permissive, yet limiting, role in Cl^- secretion in human colonic crypts.

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PC35

Structure–activity studies of fluorescein derivatives as potentiators of the CFTR Cl^- channel

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The fluorescein derivative phloxine B (2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein) is a potent modulator of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel [1,2]. Nanomolar to low micromolar concentrations of phloxine B potentiate CFTR Cl^- currents, whereas higher concentrations ($\geq 10\,\mu M$) inhibit CFTR. To understand better channel potentiation by fluorescein derivatives, we studied bengal rose B (2',4',5',7'-tetrabromo-4,5,6,7-tetraiodofluorescein), ethyl eosin (2',4',5',7'-tetrabromoeosin ethyl ester), eosin Y (2',4',5',7'-tetrabromofluorescein), TCF (4,5,6,7-tetrachlorofluorescein), DCF (2',7'-dichlorofluorescein) and fluorescein using excised inside-out membrane patches from C127 cells expressing wild-type human CFTR. We also employed molecular docking to study the interaction of fluorescein derivatives with CFTR using a head-to-tail dimer model of CFTR's nucleotide-binding domains (NBDs) [3]. When added to the intracellular solution, with the exception of TCF and fluorescein that lack halogens in the xanthene moiety of the molecule, all other fluorescein derivatives potentiated CFTR Cl^- currents with the rank order of affinity: bengal rose B (K_d, 0.32 μM) > eosin Y (K_d, 0.62 μM) > ethyl eosin (K_d, 1.72 μM) > phloxine B (K_d, 2.78 μM) > DCF (K_d, 36.24 μM). Single-channel studies demonstrated that fluorescein derivatives augment CFTR Cl^- currents by increasing open probability (P_o, $P < 0.05$, n = 6–15) with phloxine B (1 μM) and eosin Y (1 μM) both prolonging mean burst duration (MBD, $P < 0.05$) without changing interburst interval (IBI, $P > 0.05$), and DCF (20 μM) decreasing IBI ($P < 0.05$) without changing MBD ($P > 0.05$, n = 4–15). Molecular docking studies suggest that fluorescein derivatives bind at the interface of the NBD dimer primarily by hydrophobic interactions. They also suggest that the putative binding sites involve sequences from both NBD1 and NBD2 and are distinct from the two ATP binding sites. Interestingly, evaluation of the binding free energy implies that fluorescein derivatives bind tighter to NBD1. We conclude that (i) halogens in the xanthene moiety of the molecule have essential roles for CFTR potentiation and different halogens have distinct effects on channel gating; (ii) chlorines in the benzene ring of the molecule enhance potency; (iii) the carboxylic group in the benzene ring plays little role in potentiation, but is a key determinant of CFTR inhibition; and (iv) fluorescein derivatives may potentiate CFTR Cl^- channel by stabilising the formation of the NBD dimer.

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PC36

Basolateral ATP activates Cl^- secretion and Na^+ reabsorption in A6 epithelia

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The present study investigated the *in vitro* effects of extracellular ATP when applied to the basolateral border of renal epithelial cells in culture. We used the A6 epithelium cell line, derived from the distal renal tubules of the South-African clawed frog *Xenopus laevis*, as a model for the principal cells of the cortical collecting duct that exhibits both Na^+ reabsorption and Cl^- secretion in response to specific agonists. Epithelial polarisation was facilitated on permeable Anopore filter supports (pore size 0.2 μm). We continuously monitored the changes in transepithelial conductance (G_T) and short-circuit current (I_{sc}). Data are represented as means ± S.E.M. Upon the addition of ATP (5 μM) to the basolateral border, I_{sc} rapidly increased in a biphasic manner with an initial rapid increase from 1.1 ± 0.2 to 3.0 ± 0.4 $\mu A/cm^2$ with a parallel rise in G_T from 0.13 ± 0.01 to 0.15 ± 0.01 mS/cm^2 (N=6). Both increases were transient and partly recovered within 3 min. A second rise was observed for both parameters, albeit with a much slower time course, reaching a maximum ca. 20 min after the addition of ATP for G_T at 0.16 ± 0.02 mS/cm^2 and for I_{sc} at 5.5 ± 0.5 $\mu A/cm^2$. In the presence of amiloride (50 μM), the second phase of the increases in G_T and I_{sc} was completely abolished, whereas the initial rapid response remained. This indicates that the second phase of the changes in G_T and I_{sc} reflect Na^+ transport from the apical to the basolateral border. When substituting Cl^- for SO_4^{2-} in the basolateral bath, the first phase was overruled, whereas the second phase was still expressed. This is consistent with the process of Cl^- transport from the basolateral to the apical compartment during the first phase of the increases in G_T and I_{sc}. Our observations indicate that basolateral ATP elicits the immediate activation of Cl^- secretion followed by a much slower activation of Na^+ reabsorption. In a previous study (1), we observed similar responses in transepithelial transport during hypotonicity, suggesting that ATP, released across the basolateral border of the epithelium in response to cell swelling, underlies the biphasic increases of I_{sc} and G_T in these conditions (2). Further investigations are required to identify the receptors and the signal transduction pathways involved.

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

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Apical membrane targeting of ROMK2 (Kir1.1b) is independent of N-linked glycosylation

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The Kir1.1 family of inwardly rectifying K^+ channels (ROMK 1-3: Kir1.1 a-c) mediate K^+ secretion across the apical membranes of the thick ascending limb and distal nephron (Hebert et al. 2005). However, the mechanisms that determine apical membrane targeting of Kir1.1 are not understood. N-Glycans determine surface expression of a number of membrane proteins (Muth & Caplan, 2003) and Kir1.1 contains a single glycosylation consensus sequence (NXS/T) at residues 98-100. In this study, we investigated the importance of glycosylation in membrane targeting of Kir1.1b.

Filter-grown monolayers of MDCK type I cells stably expressing EGFP-Kir1.1b (WT) were treated overnight with cycloheximide (CX: 20 μg/ml) and allowed to recover for 3, 6 or 24 h either in the absence or presence of 10 μg/ml tunicamycin (TM) to inhibit glycosylation (n = 3-5 separate experiments). In a second series of experiments, monolayers of MDCK type II cells were transfected with cDNA coding for WT or a mutant (N98Q: made by QuickChange® mutagenesis) lacking the glycosylation site (n = 3-5 separate transfections). Intracellular localisation of EGFP-fusion proteins was determined by fluorescence confocal microscopy in conjunction with labelling of the apical membrane with TRITC conjugated wheat germ agglutinin (WGA) or peanut agglutinin (PNA) for MDCK I or II, respectively.

In monolayers treated with CX for 17 h, WT levels were greatly reduced and fluorescence was not evident at the apical pole of the cells. When cells were washed and incubated in CX-free media for a further 3, 6 or 24 h, the levels of fluorescence progressively recovered to control levels, and co-localised with WGA, confirming targeting of the fusion protein to the apical membrane. Following incubation with TM, the levels of WT fluorescence at 3, 6 and 24 h were similar to that of untreated controls, but, with time, the predominant species of the protein shifted from the glycosylated (~70 kDa) form to the non-glycosylated (~67 kDa) form, confirmed by SDS-PAGE and Western blotting (Ortega *et al.* 2002). Nevertheless, the fusion protein was still expressed at the apical membrane. Transient expression of MDCK II cells with either WT or N98Q, resulted in fluorescence predominantly at the apical pole of the cells, colocalising with PNA.

We conclude that polarized membrane targeting of Kir1.1b is unaffected by either prevention of the addition of complex oligosaccharides by tunicamycin, or by elimination of the N-linked glycosylation motif, when expressed in either form of MDCK cell type. These findings indicate that Kir1.1b protein is transported to the apical membrane via a mechanism(s) independent of glycosylation of the channel *per se.*

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PC38

Acute metabolic stress increases the K^+ conductance of human colonic crypts via activation of basolateral Ca^{2+}-sensitive, intermediate conductance K^+ channels (IK_{Ca})

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Gut hypoxia during major surgery is associated with increased intestinal permeability, bacterial translocation, systemic sepsis and multi-organ failure. K^+ channel activation represents a common response to metabolic inhibition in a number of different cell types. Acute metabolic stress produced by 2,4dinitrophenol (DNP) and deoxyglucose (DG) activates large conductance K^+ channels in cardiovascular tissues, and small conductance K^+ channels in liver and biliary cell lines. In the T_{84} colonic adenocarcinoma cell line, mastoparan caused a 4-fold increase in paracellular permeability that was linked to increased basolateral membrane K^+ conductance. Thus, modulation of K^+ channel activity represents a potential target for reducing the risk of systemic sepsis due to hypoxic intestinal injury.

The aim of the current study was to determine whether acute metabolic stress leads to activation of basolateral K^+ channels in human colonic crypts.

Biopsies of normal sigmoid colonic mucosa were obtained from patients undergoing routine colonoscopy. Crypts were isolated by Ca^{2+} chelation (Bowley *et al.* 2003). Whole-cell K^+ currents were measured using the perforated patch-clamp technique (0.24mg/ml amphotericin in pipette), and single channel activity in the basolateral membrane was studied in the cell-attached configuration. Cells were exposed to 100μM DNP + 5mM DG to produce metabolic inhibition. Data are presented as mean ± 1 SEM, with n, the number of experiments, and were compared by Student's paired t test.

Metabolic inhibition stimulated whole-cell currents within 5 minutes, with an increase in whole-cell conductance from 1.45 ± 0.13nS to 3.29 ± 0.19nS ($P<0.005$; n = 11), and hyperpolarization of the cell membrane voltage from -67 ± 3mV to -80 ± 3mV ($P<0.025$), consistent with K^+ channel activation; these