SA1

Dynamic regulation of epithelial transporters by PDZ-based scaffolds

M. Lee, J. Lee and K. Kim

Department of Pharmacology, Yonsei University College of Medicine, Seoul, South Korea

Aberrant ion transport caused by either hypo- or hyper-functioning of epithelial transporters, can be detrimental, and may result in life-threatening diseases, such as cystic fibrosis or secretory diarrhea. Recently, we reported biochemical and functional associations between the PDZ domain-containing protein Shank2 and a couple of epithelial transporters, the cystic fibrosis transmembrane conductance regulator (CFTR) and Na+/H+ exchanger 3 (NHE3) (1, 2). Interestingly, Shank2 attenuated the cAMP-dependent regulation of CFTR and NHE3. On the other hand, it has been previously identified that another PDZ-based scaffold EBP50 can enhance the effects of cAMP on these transporters by recruiting cAMP-dependent protein kinase anchoring protein (AKAP) (3). Shank2 and EBP50 have very similar PDZ structures that can bind to the carboxy terminus of CFTR and NHE3 (4). Therefore, Shank2 and EBP50 may compete with each other in associating with the epithelial transporters. In the present study, we determined the dynamic properties and physiological significance of these competitive interactions between PDZ-based scaffolds and epithelial transporters. In the surface plasmon resonance study, the dissociation constants (KD) of Shank2-CFTR and Shank2-NHE3 bindings were within a range similar to those of EBP50-CFTR and EBP50-NHE3, respectively, supporting the possibility of Shank2 vs. EBP50 competition in the physiological tissues. In addition, patch clamp studies revealed that the cAMP-activated CFTR chloride channel activity was dynamically regulated by the Shank2 and EBP50 competition. Lastly, in contrast to AKAP-recruiting activity of EBP50, Shank2 was found to be associated with proteins that attenuate cAMP-PKA signals. These results strongly suggest that the competitive balance between Shank2-transporter binding and EBP50-transporter binding may maintain the homeostatic regulation of epithelial ion and fluid transport.

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

SA2

Calculation of apical HCO_3^- conductance in guinea-pig pancreatic duct cells

H. Ishiguro[1], S. Naruse[2], A. Yamamoto[1], S.B. Ko[2], T. Kondo[1], M. Case[3] and M. Steward[3]

[1]Human Nutrition, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan and [3]Faculty of Life Sciences, The University of Manchester, Manchester, UK

Pancreatic duct epithelial cells secretes a HCO_3^--rich isotonic fluid into the duodenum. Recent evidence suggests that the mechanism of HCO_3^- transport across the apical membrane changes according to the anion composition of the luminal fluid. Even so, it appears to be entirely dependent on the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) (1). When luminal Cl^- concentration is high, intracellular HCO_3^- exits in exchange for luminal Cl^-, probably via one of the SLC26 family anion exchangers, and CFTR works as a Cl^- efflux pathway to maintain the inward Cl^- gradient. However, as the luminal HCO_3^- concentration rises and the luminal Cl^- concentration falls, the apical anion exchangers are no longer able to support HCO_3^- secretion, and CFTR, acting as a HCO_3^- channel, is thought to take over as the principal efflux pathway for HCO_3^- (1).

The HCO_3^- concentration of guinea-pig pancreatic juice is 140 mM or more during maximal stimulation, which is similar to human juice and much higher than in juice from mouse and rat pancreas. We previously found that the interlobular duct segments isolated from guinea-pig pancreas secreted HCO_3^- at ~0.5 nmol s^{-1} cm^{-2} (per unit area of epithelium) following secretin stimulation even when the lumen was filled with a HCO_3^--rich (125 mM) solution (2). Under these conditions the intracellular concentrations of HCO_3^- and Cl^- were ~20 mM and ~7 mM, respectively, and intracellular potential (V_m) was ~-60 mV (3). There is therefore a luminally-directed electrochemical gradient for HCO_3^- and the low intracellular Cl^- favours HCO_3^- efflux through CFTR. However, to support the observed rate of HCO_3^- secretion, an apical membrane HCO_3^- permeability of 0.25 μm s^{-1} would be required (3). In this study we have attempted to determine the apical HCO_3^- conductance by measuring changes in intracellular pH (pH_i) when the cells were de- or hyper-polarized by manipulation of extracellular K^+ ($[K^+]_B$).

Interlobular ducts (~100 μm in diameter) were isolated from guinea-pig pancreas by collagenase digestion and microdissection as described previously (2). The lumen was microperfused and pH_i was measured by microfluorometry at 37oC in ducts loaded with the pH-sensitive fluoroprobe BCECF. Dihydro 4,4' diisothiocyanatostilbene 2,2' disulphonic acid (H_2DIDS) was used to inhibit basolateral HCO_3^- transport. Changes in $[K^+]_B$ were achieved by replacement with N-methyl-D-glucamine and extracellular Na^+ was fixed at 60 mM. Averaged data are presented as the mean ± SEM. Tests for significant differences were made with Student's t test.

(1) Isolated ducts were first superfused with HCO_3^-/CO_2-free Hepes-buffered solution containing H_2DIDS (0.5 mM) and luminally perfused with a solution contaning 125 mM HCO_3^-, 24 mM Cl^-, and 5% CO_2. When $[K^+]_B$ was raised from 5 to 70 mM, pH_i in unstimulated ducts changed only slightly. During

stimulation with dibutyryl AMP (dbcAMP, 0.5 mM), depolarization caused a large increase in pH_i from 6.83 ± 0.11 to 7.32 ± 0.09 (n = 4, p < 0.01). When $[K^+]_B$ was reduced from 5 to 1 mM, pH_i decreased by 0.11 ± 0.01 (p < 0.05). (2) Experiments were also performed under Cl^--free conditions with Cl^- replaced by glucuronate. When $[K^+]_B$ was reduced from 5 to 1 mM and then raised to 70 mM in the presence of dbcAMP, pH_i decreased from 7.15 ± 0.06 (n = 4) to 7.06 ± 0.07 and then increased to 7.54 ± 0.16 (p < 0.05). (3) To calculate the HCO_3^- conductance of the apical membrane, ducts were superfused with the standard HCO_3^--buffered solution (25 mM HCO_3^-, 5% CO_2) containing H_2DIDS and dbcAMP, and the luminal solution was the same high HCO_3^- solution as previously. Apical membrane HCO_3^- fluxes were calculated from the rate of pH_i change induced by de- or hyperpolarization, taking into account the intracellular buffering capacity (4). The values of V_m, when $[K^+]_B$ was 1, 5, and 70 mM, were estimated to be -40, -50, and -60 mV using conventional microelectrodes. From the HCO_3^- flux and V_m values (and assuming a cell height of 10 μm), the HCO_3^- permeability coefficient of the apical membrane was calculated to be 0.094 ± 0.016 μm s^{-1} (n = 37).

De- and hyper-polarization caused changes in pH_i that most probably reflected the influx and efflux of HCO_3^- across the apical membrane. These HCO_3^- movements were not dependent on the presence of Cl^- and were most likely due to a HCO_3^- conductance at the apical membrane, probably CFTR. Our estimate of ~0.1 μm s^{-1} for the apical HCO_3^- permeability of guinea-pig duct cells under physiological conditions is close to the value required to account for the secretion of 140 mM HCO_3^- in this species.

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SA3

CFTR, SLC26 transporters and pancreatic HCO_3^- secretion

Y. Wang, W. Zeng, N. Shcheinikov, M. Dorward, P. Thomas, A. Soyombo and S. Muallem

Physiology, UT Southwestern Medical Center, Dallas, TX, USA

Cl^- absorption and HCO_3^- secretion is the main function of the pancreatic duct. Cl^- absorption and HCO_3^- secretion is mediated by a concerted action of Cl^-–HCO_3^- exchangers and CFTR at the luminal membrane of the duct. CFTR controls the entire process as evident from the absence of ductal Cl^- absorption and HCO_3^- secretion in CF, which results in destruction of the pancreas. The identity of the luminal Cl^-–HCO_3^- exchangers was revealed with the identification of the SLC26 transporters. The exact exact function of CFTR and the SLC26 transporters in pancreatic duct Cl^- absorption and HCO_3^- secretion and the relationship between them is not well understood. We will present evidence that two of the SLC26 transporters, SLC26a3 and SLC26a6 function as electrogenic transporters with isoform specific stoichiometry. CFTR regulates the activity of both transporters and, in turn, they regulate the activity of CFTR. Furthermore, new data suggest that the two SLC26 transporters differentially regulate CFTR. Whereas SLC26a3 only activates CFTR, SLC26a6 inhibits the basal activity of CFTR but activates the forskolin-stimulated CFTR, both in expression systems and in vivo. The significance of this form of regulation to pancreatic duct Cl^- absorption and HCO_3^- secretion is being examined in the SLC26a6-/- mouse.

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SA4

The swings and roundabouts of peptide absorption: PepT1 and NHE3

D.T. Thwaites

Institute for Cell & Molecular Biosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

The absorptive transport of many nutrients across the brush-border membrane of the small intestinal wall is mediated by cotransport through ion-coupled transport proteins. The energy which drives these movements is stored in transepithelial and transmembrane ionic gradients. The human intestinal H^+-coupled di/tripeptide transporter hPepT1 plays essential roles in nutrient (protein in the form of small peptides) absorption and in the high bioavailability of a variety of orally active peptidomimetic drugs (e.g. antibiotics, ACE inhibitors). For optimal peptide transport though hPepT1 to occur it is essential that the driving force (the transmembrane H^+ gradient) is maintained during absorption. Thus the ionic composition of the microenvironments bathing both intra- and extracellular surfaces of the brush-border membrane will control transport. Despite the fact that the isolated H^+-peptide symporter hPepT1 is a Na^+-independent transporter, dipeptide transport in intact intestinal epithelia shows a degree of Na^+-dependence. Ganapathy & Leibach (1985) proposed a model to account for this apparent Na^+-dependence where hPepT1 functions in a cooperative way with an apically-localised Na^+-H^+ exchanger (now known as NHE3). This hypothesis was tested using a range of pharmacological and physiological tools including NHE inhibitors (e.g. S1611, EIPA) and modulators of the PKA pathway (e.g. forskolin, VIP). Experiments were performed in which hPepT1 and NHE3 function were determined using intact monolayers of the human intestinal epithelial cell line (Caco-2) or hPepT1-expressing *Xenopus laevis* oocytes (Thwaites *et al.* 1999, 2002; Kennedy *et al.* 2002, 2005; Anderson *et al.* 2003). At the apical surface of Caco-2 cell monolayers, H^+-dipeptide transport via

hPepT1 led to a selective activation of NHE3 and Na^+-dependent H^+-efflux suggesting that activity of the two transporters is coupled functionally. Similarly modulators of NHE3 activity (e.g. S1611 or VIP) decreased dipeptide transport capacity across the apical membrane of Caco-2 cell monolayers but only under conditions in which NHE3 was active. In conclusion, the two transporters hPepT1 (a symporter) and NHE3 (an exchanger) function cooperatively to mediate dipeptide and Na^+ absorption across the apical membrane whilst maintaining intracellular pH and the transmembrane H^+ electrochemical gradient.

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SA5

Sensing tubular flow in the intact nephron

M.E. Juul Jensen, M.H. Christensen, H.A. Praetorius and J. Leipziger

Physiology and Biophysics, Aarhus University, Aarhus, Denmark

The mechanisms and molecules involved in renal tubular flow sensing have become a vital and exciting research area. It is the astonishing link between the central cilium found to be a flow-sensing organelle and the 'defective cilium' in different genetic diseases leading to the heterogeneous group of renal cystic diseases that drive this field. Defective tubular flow-sensing is postulated as causing the development of polycystic kidney disease (1). An increase in flow over the apical surface of cultured renal epithelia cells (MDCK) induces an increase in cytosolic calcium ($[Ca^{2+}]_i$). This 'flow response' is dependent on the presence of the central cilium (2,3). The mechanism of flow-induced $[Ca^{2+}]_i$ increases in renal epithelia remains elusive, but is postulated to involve polycystin 2 (TRPP2)-mediated influx of Ca^{2+} over the luminal membrane (4). At the same time extensive evidence indicates that in numerous biological systems including epithelia mechanical alterations promote 'non-lytic' release of nucleotides like ATP and trigger auto- and paracrine stimulation of the family of P2 receptors with subsequent increases of $[Ca^{2+}]_i$.

We therefore hypothesized that the flow-response in the renal tubule may involve mechanically-stimulated release of nucleotides. This study therefore first investigated the expression patter of luminal and basolateral P2 receptors in mouse medullary thick ascending limb (mTAL) using the P2Y2 receptor knock-out mouse. Secondly, we investigated, if a flow-induced $[Ca^{2+}]_i$ elevation can also be found in the isolated perfused intact nephron. Thirdly, it was studied, if this flow-response is affected in mice deplete of the main purinergic receptor in the mTAL.

In vitro perfusion of isolated mTAL segments and fura-2 imaging were used to record nucleotide-stimulated $[Ca^{2+}]_i$ signals. The results identify a luminal and basolateral P2Y2 receptor as the main ATP/UTP stimulated P2 receptor in this segment. Additional functional evidence suggests the presence of a basolateral P2X receptor. Subsequently, we measured mTAL $[Ca^{2+}]_i$ following changes in pressure/flow in P2Y2 receptor KO mice and their WT littermates. Pressure changes were imposed by the prompt raising of the hydrostatic pressure gradient by 80 cmH2O for 3 min. Pressure increases led to acute distension of the tubule in both WT and KO mice (by 26 and 22%, respectively). In either genotype, the increase in tubular diameter was followed by an increase in $[Ca^{2+}]_i$, as indicated by the Fura-2 ratio. This flow response was significantly larger in WT vs. KO tubules (ratio increase 0.49 ± 0.16 (n=15) vs. 0.16 ± 0.04 (n=16)). The addition of 300 μM basolateral suramin reduced the flow response in KO mice further by 65% (to 0.06 ± 0.07 (n=7)). A flow response could be elicited in the absence of bilateral Ca^{2+}. In MDCK cells ATP scavenging by bilateral apyrase (5U/ml) significantly reduced the flow response by 42%.

We also report the observation that isolated perfused mTAL tubules from P2Y2 wild-type mice display a lively $[Ca^{2+}]_i$ oscillatory behaviour. $[Ca^{2+}]_i$ oscillations were visible in video-microscopy as spontaneous 'blinking' (increase in fura-2 ratio) randomly distributed over the entire length of the tubule. A systematic analysis was undertaken to quantify spontaneous oscillatory events in mTAL of P2Y2 WT and KO mice. Spontaneous, transient $[Ca^{2+}]_i$ increases (events) above a chosen threshold of >0.1 ratio units were measured in multiple regions (20 x 25 μm) of interest of the entire tubule. In total, 1036 events with a mean frequency of one event every 6.2 min was measured in P2Y2 WT mice (n=18 tubules, total observation time: 253 min). The mean amplitude and duration of the events was 0.27 ± 0.01 ratio units and 42.2 ± 1.3 s, respectively. In sharp contrast spontaneous $[Ca^{2+}]_i$ oscillation were nearly absent in P2Y2 KO mice. A total of 138 events with a mean frequency of one event per 31.3 min were measured. Their amplitude was reduced to 0.15 ± 0.01 with no change in their duration (42.5 ± 2.9 s) (n=11 tubules, total observation time: 166 min).

These data strongly indicate that the flow-induced $[Ca^{2+}]_i$ increase in the intact renal tuble (mTAL) and cultured renal epithelial cells involves a mechanical-stimulated release of nucleotides and subsequent activation of P2 receptors. These data also indicate that spontaneous $[Ca^{2+}]_i$ oscillations in mouse mTAL require luminal and/or basolateral P2Y2 receptors and further support a mechanically stimulated, flow-dependent release of nucleotides and subsequent activation of P2 receptors.

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SA6

Effect of CFTR expression on H^+ and HCO_3^- transporters in polarized CFPAC-1 human pancreatic duct cells

Z. Rakonczay[1], P. Hegyi[1], M. Hasegawa[2], M. Inoue[2], E. Alton[3], U. Griesenbach[3], A. Da Paula[4], M. Amaral[4], B. Argent[1] and M. Gray[1]

[1]School of Biomedical Sciences, Newcastle University, Newcastle upon Tyne, UK, [2]DNAVEC Corporation, Tsukuba, Japan, [3]Dept. of Gene Therapy, Imperial College and The UK CF Gene Therapy Consortium, London, UK and [4]Dept. of Chemistry & Biochemistry, University of Lisboa, Lisbon, Portugal

Pancreatic duct cells (PDCs) secrete an alkaline, HCO_3^--rich fluid, and this process is markedly reduced in cystic fibrosis as a result of dysfunction in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane chloride channel. However, it is still unclear the exact role that CFTR plays in HCO_3^- transport by PDCs. The initial step of HCO_3^- secretion is the uptake of HCO_3^- into the duct cells from the extracellular space. HCO_3^- can enter the epithelium either by the forward transport of HCO_3^-, via a basolateral Na^+/HCO_3^- co-transporter, or by the diffusion of CO_2 into the cells, subsequent hydration to H_2CO_3 by carbonic anhydrase, and backward transport of protons via Na^+-H^+ exchangers and/or H^+ pumps. HCO_3^- secretion across the apical membrane is thought to occur via CFTR and the SLC26 anion exchangers, SLC26A3 (DRA) and SLC26A6 (PAT-1). However, the relative importance of each of these apical transporters in HCO_3^- secretion is a controversial issue (Argent et al. 2006; Steward et al. 2005). Thus it is still unclear whether CFTR's main role in HCO_3^- secretion is to secrete HCO_3^- directly, to provide a source of luminal Cl^- to support apical Cl^--HCO_3^- exchange or to activate the apical $Cl^{--HCO_3^-}$ exchangers. It remains possible that all three mechanisms are utilized by the cells, perhaps under different physiological situations.

To gain further insight into this issue we have been investigating the effects of CFTR expression on H^+ and HCO_3^- transporters involved in ductal HCO_3^- secretion. To do this we have developed a novel system for efficient gene transfer of CFTR to polarized monolayers of CFPAC-1 cells (originally derived from a cystic fibrosis patient), using a recombinant Sendai virus construct (Yonemitsu et al. 2000), which does not impair cell polarity. This has allowed us to examine H^+ and HCO_3^- transport activities in polarized CFTR-deficient and CFTR-expressing CFPAC-1 cells.

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SA7

The role of anion exchangers in bicarbonate-secreting epithelia

M.C. Steward

Faculty of Life Sciences, University of Manchester, Manchester, UK

Work in this and other laboratories over the last ten years has led to a better understanding of the mechanism of HCO3- secretion in the pancreatic duct, and particularly the role of CFTR in this process (Steward et al. 2005). It has also become clear that it is the loss of HCO3-, rather than Cl-, secretion that underlies much of the pathology of cystic fibrosis in the pancreas and in other affected tissues. Despite recent progress, a fundamental question remains unanswered: how is HCO3- secreted across the apical membrane at maximal rates when the luminal (secreted) concentration may exceed 140 mM? More specifically, does the CFTR channel itself provide the main route for HCO3- secretion across the apical membrane (Shcheynikov et al. 2004)? Or is the role of CFTR to promote the activity of a physically linked Cl—HCO3- exchanger (Ko et al. 2004)?

In the pancreatic duct the apical anion exchanger is probably a member of the recently discovered SLC26 family (Mount & Romero, 2004). This is certainly involved in the initial stages of the secretory process when there is still a significant amount of Cl- present in the lumen. However, even allowing for the possibility that it might be electrogenic, it is difficult to see how such an exchanger could operate maximally when the luminal HCO3- concentration is as high as 140 mM. On the contrary, recent work on the pancreatic duct suggests that HCO3- is more likely to be secreted via an apical anion conductance under these conditions, most probably CFTR (see Ishiguro et al., this symposium).

Previous work with the Calu-3 cell line, which is a good model for the serous cells of human airway submucosal glands, has also suggested that CFTR may act as a HCO3- channel and that this may be the main pathway for HCO3-, as well as Cl-, secretion in these cells (Hug et al. 2003). Nonetheless, our recent work suggests that an anion exchanger is also present in the apical membrane (Moore et al. 2005), that it is activated by CFTR, and that it shows many of the features of the SLC26 family. If so, this cell line may prove to be a useful experimental model for investigating the interactions between CFTR and the SLC26 exchangers during HCO3- secretion.

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SA8

Time-lapse DIC studies of airway submucosal gland secretion

J. Wine

Stanford University, Stanford, CA, USA

Cystic fibrosis (CF) is caused by the malfunctioning of CFTR, an anion channel that either directly or indirectly mediates Cl^-, and HCO_3^--mediated fluid secretion in many exocrine organs, including the submucosal glands of the airways. People with CF have defective lung defenses, and so develop chronic lung infections with bacteria and fungi that are localized in static mucus within the airways (for review see Wine & Joo, 2004). Because glands produce most airway mucus, we developed optical methods to quantify secretion by individual glands (Joo et al. 2001), and discovered that the glands of people with CF no longer secrete in response to VIP or forskolin (Joo et al. 2002). The glands also show more subtle defects to cholinergic stimulation (Jayaraman et al. 2001).

To better understand how glands elaborate mucus, we initiated studies of gland secretion using differential interference contrast (Nomarski or DIC) microscopy. These studies can potentially localize the cellular origins of various kinds of secretion and can give a host of new insights in gland function.

As one example, immunohistochemistry has localized CFTR in two different regions of the glands: either primarily in the serous cells (Engelhardt et al. 1992) or primarily in the ciliated ducts (Kreda et al. 2005). To test the hypothesis that the ducts, rather than the acini, might be the origin of most CFTR-mediated fluid secretion, we used Nomarski DIC microscopy to track the flow rates of particles within the mucus of forskolin-stimulated glands. Within the ducts, particles flowed at a constant rate, indicating that neither secretion nor absorption was occurring, but in the acini they accelerated, indicating secretion. This was confirmed by micro-ligation experiments of isolated glands. When the junction between the unbranched duct and the rest of the gland was ligated and the gland was stimulated, no mucus exited the duct orifice, but the acinar region swelled. With double ligations that isolated the ducts, the acinar region swelled after stimulation but the duct neither swelled nor contracted.

These results support the functional location of CFTR in acinar serous cells, but they leave open the function of CFTR in the duct. One possibility is that CFTR (and ENaC) in the ducts are primarily absorptive, and then in cystic fibrosis glands, hyperabsorption via unregulated, overactive ENaC might alter mucus in the duct so that its secretion is blocked. To test that we applied ENaC blockers to normal and CF glands, but these did not alter the volume of gland secretion, supporting the proposal that hyposecretion, not hyperabsorption, is the basic defect of CF glands (Joo et al. 2006).

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SA9

Primary cilia and renal cyst formation in a mouse model of nephronophthisis-type 2

B. Verdon[1], M.A. Gray[1], N.L. Simmons[1], J.A. Sayer[1], J.A. Goodship[2], T. Strachan[2] and B. Argent[1]

[1]Institute for Cell & Molecular Biosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, UK and [2]Institute of Human Genetics, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

Nephronophthisis-type 2 (NPHP2) is an infantile renal disease whose features include: tubular basement membrane disruption, renal interstitial fibrosis, enlarged kidneys and widespread renal cyst development (Otto *et al.* 2003). Occasionally, reversal of the left-right body plan (*situs inversus*) also occurs. NPHP2 is caused by a mutation of the *INVS* gene located on human chromosome 9q21–q22 (Otto *et al.* 2003). In the mouse, deletion of exons 3–11 of the *Invs* gene also causes a phenotype of cystic kidneys and *situs inversus*. Detailed anatomical studies of the *inv* -/- mouse kidney have confirmed that it is a good model for the early stages of human NPHP2 (Phillips *et al.* 2004).

The protein encoded by the *INVS* (human) and *Invs* (mouse) genes is called inversin (for a review see Eley et al. 2004). The full length murine protein contains 1062 amino acids, but shorter isoforms with 924 and 897 amino acids have been identified. Analysis of the human *INVS* gene sequence suggests that the human and mouse inversin proteins are very similar (Eley *et al.* 2004). Inversin is expressed in the plasma membrane and junctional complexes of renal epithelia, and is highly expressed in the primary cilium on renal cells. Many human and murine proteins, whose mutation is associated with renal cyst formation and, in some cases laterality defects, are also expressed in the primary cilium. Therefore, establishing inversin's function in the primary cilium is probably the key to understanding why humans with NPHP2 and *inv* -/- mice develop renal cysts.

Renal cyst formation probably involves both abnormal morphogenesis and enhanced secretory activity by the tubular epithelium. Inversin expressed in the primary cilium may affect morphogenesis by acting as a flow-regulated molecular switch between canonical and noncanonical Wnt signalling pathways, which regulate a diverse range of developmental processes (Simons *et al.* 2005). The noncanonical Wnt pathway may be important for correct development of the renal tubules and for the maintenance of correct cell orientations in developed tubules. In the absence of inversin, the canonical Wnt signalling pathway operates unabated, leading to the cystic phenotype characteristic of NPHP2 and the *inv* -/- mouse (Germino 2005; Simons *et al.* 2005).

Our work is concerned with the secretory aspects of cyst formation. The purposes of this study were: (i) to establish whether secretory Cl^- channels (CFTR and Ca^{2+}-activated Cl^- channels)

were upregulated in *inv* -/- cells, and (ii) to test whether Ca^{2+} signalling, including cilium-mediated Ca^{2+} signalling, was abnormal in the renal cells of *inv* -/- mice.
Renal collecting ducts were isolated from wild type (+/+), heterozygote (+/-) and homozygous *inv* (-/-) mice and used to produce primary cultures of renal collecting duct cells. Most of the cultured cells possessed a primary cilium, which was clearly visible using differential interference contrast optics. It was possible to bend the cilium on a single cultured cell using either positive or negative pressure applied via a micropipette. The results of experiments in which we measured whole cell Cl^- currents and global changes in $[Ca^{2+}]_i$ following application of agonists and bending of the primary cilium will be discussed.

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SA10

SLC4 and SLC26 polypeptides: anion exchangers in flux

S.L. Alper, J.S. Clark, C.E. Kurschat, B.E. Shmukler, D.H. Vandorpe and A.K. Stewart

Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Plasmalemmal Na+-independent Cl-/HCO3- exchangers regulate intracellular pH, [Cl-], and cell volume. In polarized epithelial cells they contribute also to transepithelial secretion and reabsorption of acid-base equivalents and of Cl-. These transporters are encoded by members of the two evolutionarily unrelated mammalian gene families, SLC4 and SLC26. Human SLC4A1/AE1 mutations cause either the erythroid disorders spherocytic hemolytic anemia or ovalocytosis, or distal renal tubular acidosis. SLC4A2/AE2 knockout mice die at weaning. Human SLC4A3/AE3 polymorphisms have been associated with seizure disorder. Mutations in 4 of the human SLC26 genes cause chondrodysplasias, congenital diarrhea, deafness, and hypothyroid goiter.
Although mammalian SLC4/AE polypeptides mediate only electroneutral Cl-/anion exchange, the structural and mechanistic differences between electroneutral and electrogenic anion transport can be subtle. Thus, the electroneutral trout AE1 anion exchanger also promotes or mediates increased anion conductance and uncoupled osmolyte transport. The presence of the electroneutral anion exchanger mouse AE1 in the erythrocyte membrane is required for DIDS-sensitive erythroid Cl- conductance (although definitive evidence for AE1 mediation of Cl- conductance is lacking). A single missense mutation allows mouse AE1 to mediate both electrogenic SO42-/Cl- exchange or electroneutral, H+-independent SO42-/ SO42- exchange.
SLC4 anion exchangers appear to be homooligomers, but also function with the assistance of auxiliary subunits. Thus, glycophorin A and protein 4.2 act as subunits of erythroid AE1 affecting trafficking and transport rate. Depending on the expression system, direct binding of carbonic anhydrases bySLC4 anion exchangers is either stimulatory or obligatory for HCO3- transport. In the Xenopus oocyte, the AE1 C-terminal cytoplasmic tail residues thought to bind carbonic anhydrase II are dispensable for Cl-/Cl- exchange, but required for Cl-/HCO3- exchange. Membrane-associated ecto-carbonic anhydrases also bind to SLC4 anion exchangers and modulate their activity.
Cellular pH regulation by SLC4 anion exchangers implies that anion exchanger activity is itself pH-sensitive. However, SLC4/AE1 is remarkably insensitive to pH over the physiological range, whereas SLC4A2/AE2 is acutely and independently inhibited by intracellular and extracellular H+. This regulation requires integrity of the most highly conserved sequence of the AE2 N-terminal cytoplasmic domain, along with adjacent residues in which individual missense mutations acid-shift pHo sensitivity of AE2. These regulatory determinants together are modeled to form contiguous surface patches on the AE2 cytoplasmic domain. In contrast, the N-terminal variant AE2c polypeptide exhibits an alkaline-shifted pHo-sensitivity, as do certain transmembrane domain His mutants. A novel, non-conserved region of the AE2 transmembrane domain has been recently shown to be required for pH-sensitivity. AE2-mediated anion exchange is also stimulated by ammonium and hypertonicity via Ca2+-dependent pathways.
In contrast to SLC4 anion exchangers, the SLC26 anion transporter polypeptides exhibit remarkable sequence diversity among near-species orthologs, most marked among SLC26A6 polypeptides. This sequence diversity prompted systematic functional comparison in Xenopus oocytes of mouse slc26a6 with human SLC26A6 variants. Whereas these orthologs exhibited similar rates of bidirectional 14C-oxalate flux and of Cl-/HCO3- and Cl-/OH- exchange subject to similar stimulation by CFTR, they differed markedly in rates of Cl- exchange for trans-Cl-, sulphate, and formate. Studies with mouse-human chimera slc26a6 cRNAs showed that high transport rates cosegregated with the transmembrane domain of mouse slc26a6, and that multiple parts of the transmembrane domain contribute to the phenotype.
We found that both species orthologs mediated electroneutral Cl-/HCO3- and Cl-/OH- exchange as measured by two electrode voltage clamp. In contrast, Cl-/oxalate exchange by mouse slc26a6 was electrogenic. Isotopic flux studies performed under voltage clamp conditions confimed these observations.
Although studies of mouse SLC26 knockout models are revealing physiological roles of these transporters in secretory processes for bicarbonate and other substrate anions, the substantial sequence differences between orthologous mouse and human transporter polypeptides correlate with species-specific differences in transport properties. These intrinsic differences must be considered when extrapolating results from the mouse to human pathophysiology. Delineation of the physical basis for the unusual species-specific differences in anion selectivity of slc26a6 polypeptides should also provide general insight into structure-function relationships of anion binding and translocation.

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SA11

Channel or transporter? The dichotomy in a family of double-barrelled chloride transport proteins

M. Pusch

Istituto di Biofisica, Genoa, GE, Italy

In mammals, nine genes code for Cl transporting CLC proteins. Several CLCs are involved in human genetic diseases. Triggered by the finding of Accardi & Miller (2004) that the bacterial CLC-ec1 is a secondary active, highly electrogenic, Cl-proton exchanger, we investigated if CLC-5, that is mutated in Dent's disease, could similarly be a Cl-proton exchanger. We found that indeed the strongly outwardly rectifying current carried by CLC-5 is associated with a significant movement of protons. Detailed measurements, employing pH-sensitive microelectrodes, allowed us to conclude that CLC-5 and CLC-4 function as Cl-proton antiporters, while other CLCs (CLC-0, CLC-2, CLC-Ka) do not transport protons to a significant amount and are thus passive ion channels. While this finding is important to understand the physiological role of CLC-5 in endocytosis, the precise physiological function of CLC-5, and a possible direct role in vesicular acidification, remain speculative.

Analysis of the CLC-0 mutation E166D, in which the 'gating glutamate' is substituted by Asp shed further light on the relationship between the channel and the transporter character of CLC proteins, pointing to an important role of protonation of this acidic residue from the intracellular solution as a major source of voltage dependence of channel function.

In parallel studies we investigated pharmacological properties of renal CLC-K/barttin channels. Previously, we had identified a blocking site on CLC-Ka that is located in the extracellular pore mouth. Two amino acids (N/D-68 and G/E-72 in CLC-Ka and CLC-Kb, respectively) are critical to confer strong block by DIDS and 3-phenyl-CPP of CLC-Ka and weak block of CLC-Kb. Surprisingly, we now found that niflumic acid (NFA) increases CLC-Ka/Kb currents in the 10-1000 μM range. Flufenamic acid derivatives or high concentrations of NFA inhibited CLC-Ka, but not CLC-Kb, or blocker-insensitive CLC-Ka mutants, indicating that the activating binding site is distinct from the blocker binding site. These molecules provide a starting point for identification of diuretics or drugs useful in the treatment of Bartter's syndrome.

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SA12

Distribution of ion transporters in the choroid plexus – an atypical epithelium

J. Praetorius

Institute of Anatomy, The Water and Salt Research Center, University of Aarhus, Aarhus, Denmark

The choroid plexus epithelium secretes electrolytes and fluid into the brain ventricular system at high rates; comparable or exceeding the reabsorption rate of the renal proximal tubule. However, the mechanism by which the cerebrospinal fluid (CSF) is secreted is greatly different than in other fluid-secretory tissues e.g. the salivary or sweat glands. Studies of the mammalian choroid plexus suggest that the pivotal event in CSF secretion is the active transport of Na^+ from the epithelial cell to the CSF, mediated by the apically positioned Na^+,K^+-ATPase (1). The apical Na^+,K^+,$2Cl^-$ cotransporter, NKCC1, may also contribute to the Na^+ secretion and to recycling of K^+ to the CSF (2) in concert with apical K^+ channels (3). Cl^-, however, is mainly secreted through electrogenic apical mechanisms, probably involving one or more Cl^- channels (4). Water, in turn, would follow by a transcellular pathway, i.e. through the water channel AQP1 (5) or by a paracellular pathway. The Cl^-/HCO_3^- exchanger, AE2, originally cloned from the rat choroid plexus (6), serves as a base extruder to maintain intracellular pH in many epithelia. AE2 may also contribute to basolateral Cl^- uptake in the choroid plexus.

The basolateral cellular entry mechanisms for Na^+ and H_2O into the choroid plexus are less well defined. The mRNA encoding the Na^+/H^+ exchanger, NHE1, is expressed in the choroid plexus of rat, and amiloride sensitive transport has also been detected several times by e.g. Conrad E. Johanson's group. In addition, a basolateral DIDS sensitive Na^+ and CO_2/HCO_3^- dependent mechanisms seem to be involved in secretion in the presence of CO_2/HCO_3^- (7, and others). Recently, it was suggested that the basolateral uptake of Na^+ may be mediated by a Na^+ dependent Cl^-/HCO_3^- exchanger, NCBE. This transporter is expressed exclusively in the basolateral plasma membrane domain of epithelial cells in the choroid plexus epithelium and may explain the DIDS sensitive Na^+:HCO_3^- cotransport in these cells. An electroneutral Na^+:HCO_3^- cotransporter, NBCn1, is also expressed in the basolateral membrane of choroid plexus epithelial cells. NBCn1 is not likely to play a major role in the transepithelial movement of Na^+ and HCO_3^- as it does not display the DIDS sensitivity of the main Na^+ entry mechanism.

The CSF secretion mechanism in humans is thought to be similar to the rodent system; however, few previous studies have addressed the molecular basis for choroid plexus transport in humans. Therefore, we aimed to localize several of the aforementioned proteins to the human choroid plexus. The Na^+,K^+-ATPase α1-subunit is localized apically in the human choroid plexus epithelial cells, as was NKCC1. AQP1 is predominantly situated in the apical plasma membrane domain, although weaker basolateral immunoreactivity is also observed. AE2 is localized basolaterally, as is NCBE and NBCn1. No NHE1 immunoreactivity is found. Hence, the human choroid plexus epithelium displays an almost identical distribution pattern of water channels and Na^+ transporters as the rat and mouse choroid plexuses (Fig. 1).

In conclusion, it remains to be determined how Na^+ enters the choroid plexus epithelium, how Cl^- and HCO_3^- exit the cells. Moreover, precise estimates of the ionic gradients and subcellular localization of the involved transporters are warranted to better model the CSF secretory process.

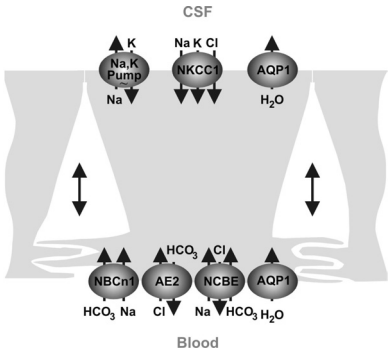

Figure 1. Model showing the localisation of the Na^+,K^+-ATPase, AQP1, NKCC1 and HCO_3^- transporters in the human choroid plexus based on the present and previous observations. The water channel AQP4, the Na^+/H^+ exchanger NHE1, and the electrogenic Na^+-base cotransporters NBCe1 and NBCe2 were not detected in the human choroid plexus.

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SA13

Use of knockout mice to dissect the role of K^+ channels in submandibular gland function

V.G. Romanenko[2,1], N. Tetsuji[1], T. Begenisich[2,1] and J. Melvin[2,1]

[1]The Center for Oral Biology in the Aab Institute of Biomedical Sciences, University of Rochester, Rochester, NY, USA and [2]Department of Pharmacology & Physiology, University of Rochester School of Medicine & Dentistry, Rochester, NY, USA

Salivary gland acinar cells express both intermediate- and large-conductance Ca^{2+}-activated K^+ channels, often termed IK (Sk4) and BK (Slo) channels, which are encoded by the *Kcnn4* and the *Kcnma1* genes, respectively. To test the roles of these channels in submandibular glands, we examined the functional consequences of null mutations in the *Kcnn4* and the *Kcnma1* genes. As anticipated, we found that functional IK1 and BK channels were absent in submandibular acinar cells from *Kcnn4*$^{-/-}$ and *Kcnma1*$^{-/-}$ mice, verifying their genetic identities. The cytoplasmic Cl^- concentration of salivary acinar cell is approximately 4- to 5-fold higher than the Cl^- electrochemical equilibrium. Thus, when Ca^{2+}-mobilizing receptor agonists stimulate the apical Ca^{2+}-activated Cl^- channel, Cl^- exit occurs into the lumen and Na^+ and water follow. However, Cl^- exit also depolarizes the acinar cell, and if the membrane potential approached the Cl^- equilibrium potential, net Cl^- movement and fluid secretion would stop. Secretion depends, therefore, on the maintenance of a membrane potential (V_m) negative to the Cl^- reversal potential. This membrane hyperpolarization has been linked to IK1 and/or BK channels. As expected, stimulation of acinar cells from wildtype mice with the muscarinic receptor agonist carbachol (0.3 μM) produced a rapid depolarization to near the Cl^- reversal potential, but the V_m quickly returned to a point approximately midway (-55±2 mV) between the equilibrium potentials for K^+ (E_{eq}=-85 mV) and Cl^- (E_{eq}=-24 mV). Unexpectedly, submandibular acinar cells from either *Kcnn4*$^{-/-}$ or *Kcnma1*$^{-/-}$ mice maintained a relatively hyperpolarized V_m during muscarinic receptor stimulation (-51±3 and -48±3 mV for *Kcnn4*$^{-/-}$ and *Kcnma1*$^{-/-}$, respectively) – values comparable to that seen in wildtype mice. Consistent with this observation, fluid secretion induced *in vitro* by 0.5 μM carbachol was not inhibited in mice lacking expression of either IK1 or BK channels in isolated, perfused submandibular glands. These results indicate that in isolation, either IK1 or BK channels can maintain the hyperpolarized V_m required for normal sustained fluid secretion. In contrast, the V_m of acinar cells in double *Kcnn4*$^{-/-}$/*Kcnma1*$^{-/-}$ mice was significantly depolarized (-35±2 mV), and fluid secretion was markedly reduced (~70%). Thus, in the absence of both IK1 and BK channels, acinar cell V_m remained sufficiently hyperpolarized to support fluid secretion, but at a significantly reduced rate. We found that inhibition of the electrogenic Na^+,K^+-ATPase produced little, if any, depolarization during muscarinic receptor stimulation in wildtype acinar cells (-51±5 mV), whereas simultaneous inhibition of both Ca^{2+}-activated K channels *and* the ATPase depolarized cells to near the Cl^- reversal potential (-27±4 mV). Taken together, our results demonstrate that IK1 and BK channels play major roles in submandibular acinar cell function, and under severe pathological conditions (such as loss of expression of IK1 and BK channels) Na^+,K^+-ATPase has a limited capacity to hyperpolarize V_m sufficiently to support secretion.

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SA14

Function and morphology of the paracellular route for salivary secretion

M. Murakami[1], A. Segawa[2], A. Hill[3], B. Sachar-Hill[3], S. Hashimoto[4], K. Hosoi[5] and K. Murdiastuti[5]

[1]Nano-Str Physiol, Natl Inst Physiol Scis, Okazaki, Japan, [2]Anatomy, Kitasato Univ, Sagamihara, Japan, [3]Physiological Laboratory, Cambridge Univ, Cambridge, UK, [4]Pathology, Tokyo Dental College, Chiba, Japan and [5]Mol Oral Physiology, Inst Healthbiosci, Univ Tokushima Grad Sch, Tokushima, Japan

Across epithelia the water is secreted or absorbed through the cell membrane (transcellular pathway) or the tight junction (paracellular pathway), each route involving specific molecules such as aquaporin or claudin. The paracellular route also allows the passage of some substrates from the circulation depending upon the composition of serum. This idea has fascinated many scientists since long ago. The salivary sugar was recognized as an indicator of blood sugar before the discovery of insulin, but had no clinical application. Modern techniques should be developed for blood-free clinical tests in the near future.

The filter size of the paracellular route was estimated by perfusion with various sizes of radioactive dextran in the isolated perfused submandibular gland and analysis reveals that a high proportion of the water crosses by the paracellular route (Murakami et al. 2001). Then discrimination of transcellular and paracellular fluid had been difficult until Segawa noticed the difference in Lucifer Yellow (LY) fluorescence of intercellular canaliculi (IC) between the whole perfused gland and the acini isolated enzymatically. In the latter case, when the enterotoxin (contaminated in the collagenase preparation) bound to claudin and 'paralyzed' the tight junction (TJ). Application of carbachol decreased the fluorescence in IC, indicating that the LY was diluted by fluid from the cytosol. We estimated transcellular fluid movement into the lumen due to dilution of LY in the IC of isolated acini (Segawa et al. 2002). By subtraction of this estimate from the whole fluid secretion, we could assess the time course of development of paracellular fluid transport by muscarinic stimulation. The paracellular component started to increase slowly after 30 s from start of transcellular secretion and reached the plateau levels around 1 min after start of transcellular output. The plateau level was higher than that of the transcellular fluid secretion level.

How is the paracellular route modulated? During parasympathetic stimulation, addition of sympathetic stimulation increased the passage of sulphhate (Martin & Burgen, 1962), suggesting that the paracellular passage is activated by addition of b-adrenergic stimulation. This finding was reproduced by using LY during perfusion of the rat submandibular gland. To study the underlying mechanism for opening the paracellular route, we observed the TJ structure using freeze-fracture (FF) replicas on rapidly frozen tissues with liquid helium (Hashimoto et al. 2003). During combined stimulation with carbachol and isoproterenol (isoprenaline), microvilli disappeared, and the secretory granule fused with the plasmalemma. The meshwork of TJ strands were rearranged more irregularly and became more straggled. Furthermore, the alignment of TJ membrane particles was interrupted at several points and free ends and terminal loops appeared at the basal side. These changes in the TJ may be related to the subcellular structural modulation of the actin filament network and associated TJ proteins.

Changes in fluid transport rates created by hyperosmolar sucrose during the perfusion of isolated rat submandibular glands (SMG) in vitro reduce secretion rates much more than predicted by the osmotic theory of fluid production. However, these are in accord with a theory involving AQP5 feedback control of paracellular fluid transfer (Hill & Shachar-Hill, 2006). The changes in transport rate can be predicted with parameters determined earlier for this gland (Murakami et al. 2001) and a model of the SMG system is presented. Experiments were performed with SMG from genetically selected rats that have very low levels of AQP5 as determined by Western blotting (Murdiastuti et al. 2002). The fluid secretion rates after osmolarity changes were those expected for the osmotic theory. We suggest that control of paracellular flow has been lost in these low AQP5 rats, which have reverted to osmotic fluid production. Retrograde injection of Hg ions into the duct of normal rats partially inhibited AQP5, leading to a concentration-dependent reduction in flow rates. However, reduction of fluid secretion after osmolarity changes was still close to that of normal rats. The results suggest the involvement of a feedback loop including AQP5 and paracellular fluid transport.

The meshwork structure of TJ is simple in the salivary gland; to link the data from single cells to the whole organ, we must include the function of the paracellular route in the salivary gland.

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SA15

Interactions of CFTR and anion exchangers in the murine duodenum

L.L. Clarke

Biomedical Sciences, Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO, USA

Bicarbonate transport across the duodenal epithelium establishes an alkaline mucus layer that protects the epithelium from gastric acid and provides an environment favorable to efficient nutrient digestion/absorption. The process of transepithelial HCO_3^- secretion involves the concerted activities of CFTR and apical membrane anion exchangers, including Slc4a9 (anion exchanger 4, AE4) and two members of the multifunctional anion exchanger family, Slc26a3 (down-regulated in adenoma, DRA) and Slc26a6 (putative anion transporter-1, PAT-1) (1,2).

Studies of transepithelial HCO_3^- secretion using wild-type (WT) and CFTR(-) murine duodena have shown that basal secretion

is reduced in the absence of CFTR and that the process is primarily mediated by Cl^-/HCO_3^- exchangers because secretion is abolished by removal of luminal Cl^-. During cAMP stimulation, secretion is apparently dependent on a CFTR-mediated HCO_3^- conductance because secretion is essentially unaffected by luminal Cl^- removal and abolished in the absence of CFTR. However, important exceptions to the dominance of a CFTR HCO_3^- conductance during cAMP-stimulated secretion have been identified whereby ~50% of cAMP-stimulated HCO_3^- secretion is not associated with the short-circuit current (Isc), i.e. electroneutral (3). To further investigate the interactions of CFTR and the anion exchangers in native intestine, we measured intracellular pH in the villous epithelium of murine duodenum placed in a horizontal Ussing chamber. Contributions of the specific anion exchangers were investigated using mice with gene-targeted deletions of AE4, DRA, PAT-1 and/or CFTR.

Upper villous epithelium. Earlier studies proposed that Cl^- channels enhance the activity of anion exchangers by providing a "leak" pathway that sustains the Cl_{out}:Cl_{in} concentration gradient. More recent studies suggest that CFTR directly associates with anion exchange proteins to increase their activity (4). In studies of the upper villous epithelium, we found that the basal rate of Cl^-/HCO_3^- exchange was significantly reduced in the CFTR(-) duodenum. Ion substitution revealed that greater transport rates in WT duodenum were detected only when the anion exchanged for HCO_3^- was CFTR-permeable. Further, glybenclamide block of CFTR reduced the rate of Cl^-/HCO_3^- exchange in WT to a level equivalent with CFTR(-) epithelium. Thus, the anion channel activity of CFTR rather than its mere presence in the apical membrane is required to promote basal Cl^-/HCO_3^- exchange. Studies of anion exchanger knockout mice demonstrated that Cl^-/HCO_3^- exchange was unchanged in the AE4(-) duodenum. In contrast, anion exchange was decreased by ~20% in the DRA(-) and ~80% in the PAT-1(-) duodenum, respectively. It was concluded that CFTR indirectly facilitates the activity of PAT-1 in the villous epithelium by providing a Cl^- leak pathway. DRA may not participate due to coupling with the Na^+/H^+ exchanger, NHE3, for NaCl absorption.

Lower villous epithelium. Studies comparing WT and knockout duodena revealed that Cl^-/HCO_3^- exchange in the lower villous epithelium was not altered in the PAT-1(-) mice, but completely abolished in DRA(-) mice. Paradoxically, removal of luminal Cl^- in the DRA(-) lower villus caused cell acidification, which was CFTR-dependent because luminal Cl^- removal did not alter the pH_i in the DRA(-)/CFTR(-) duodenum. The outcome of these experiments is consistent with a report that the HCO_3^- permeability of CFTR is increased by removal of extracellular Cl^- (5).

Contributions of PAT-1 and DRA to transepithelial HCO_3^- secretion. pH stat measurements of PAT-1(-) duodenum revealed that PAT-1 only contributes to basal (~25%) but not cAMP-stimulated HCO_3^- secretion. In contrast, DRA was responsible for ~50% of both basal and cAMP-stimulated HCO_3^- secretion in physiological (i.e. Cl^- containing) medium. The cAMP-stimulated Isc response was unchanged in the DRA(-) intestine, indicating normal CFTR activity. The remaining component of stimulated HCO_3^- secretion can be ascribed to a CFTR-mediated HCO_3^- conductance, which would comprise ~15% of the stimulated Isc, i.e. approximating the HCO_3^-:Cl^- permeability ratio of CFTR. Interestingly, cAMP-stimulated HCO_3^- secretion was normalized in the DRA(-) duodenum by luminal Cl^- removal, again suggesting that the CFTR-mediated HCO_3^- conductance is enhanced in the absence of extracellular Cl^-.

In summary, in the upper villous epithelium where expression is low, CFTR plays a passive role as a Cl^- leak pathway that facilitates the activity of PAT-1. In the lower villous epithelium, CFTR's association confers the ability for cAMP stimulation to DRA. Understanding the roles that PAT-1 and DRA play in duodenal HCO_3^- secretion may lead to therapies normalizing juxtamucosal pH to correct deficiencies of nutrient digestion and absorption in the CF intestine.

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SA16

Coupling between water and substrate transport in epithelial cotransporters and uniports

T. Zeuthen, N. MacAulay and E. Zeuthen

Nordic Centre for Water Imbalance Related Disorders. Institute of Medical Physiology, University of Copenhagen, Copenhagen, Denmark

The general mechanism of transepithelial water transport remains unexplained. Uphill transport of water across epithelia, for example, cannot result from osmotic mechanisms; the driving forces are directed the wrong way. Such problems are circumvented in epithelial models which combine osmotic transport i.e. in aquaporins with direct coupling of water and substrate transport in cotransporters.

The relations between substrate and water transport were studied in Na^+-coupled cotransporters of glucose (SGLT1) and of iodide (NIS) expressed in Xenopus oocytes and compared to that of the uniport GLUT2. The water transport was monitored from changes in oocyte volume at a resolution of 20 pl, more than one order of magnitude better than previous investigations (Zeuthen et al. 2005). The rate of cotransport was monitored as the clamp current obtained from two-electrode voltage clamp. The data demonstrated a fixed ratio between the turn-over of the cotransporter and the rate of water transport under a variety of experimental procedures: isosmotic application of substrate, rapid changes in clamp voltage, or poisoning. Transport of larger substrates gave rise to less water transport: For the rabbit SGLT1 about 380 water molecules were cotransported along with the 2 Na^+ ions and the glucose analogue α-MDG (MW 194), using the larger sugar arbutin (MW 272) the water coupling ratio was reduced by a factor of about 0.85. For the human SGLT1 the respective numbers were 235 and 0.85. For NIS about 250 water molecules were cotransported for each 2 Na^+ and 1 thiocyanate (SCN^-, MW 58), with I^- as anion (MW 127) only 160 water molecules. The effect of substrate size supports a molecular mechanism for water cotransport and is opposite to what would be expected from unstirred layer effects. Fluxes into the oocyte via

GLUT2 showed that diffusion of sugar in the cytoplasm did not give rise to significant unstirred layer effects; a similar conclusion has been reached for transport of Na^+ ions maintained by the ionophore gramicidin.
We conclude that coupling between water and substrate transport takes place by a molecular mechanism in the cotransporter itself. An epithelial model based upon the Na^+/glucose and K^+/Cl^- cotransporters and the GLUT2 uniport is presented; the K^+/Cl^- cotransporter has previously been shown to cotransport about 500 water molecules per turnover (Zeuthen, 1994; Zeuthen & MacAulay, 2002). The ability of the epithelial model to transport water uphill is discussed.

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SA17

The post-genomic world of UT-A urea transporters

C.P. Smith

Faculty of Life Sciences, The University of Manchester, Manchester, UK

From bacteria to mammals, specialized membrane proteins regulate the transport of urea across cellular membranes. In mammals these proteins are derived from either the UT-A (Slc14a2) or the UT-B (Slc14a1) genes. Proteins arising from both genes show a broad distribution throughout the body indicative of multiple physiological roles. In this presentation I will focus on UT-A urea transporters with special emphasis on recent findings.
The structure of the UT-A gene has been resolved in mouse, rat and human and resides on Chromosome 18 q12.3. Not surprisingly, these three genes share several characteristics. These include; their large size (all of which are greater than 400kb); they reside in tandem on Chromosome 18 with the UT-B gene; their transcription is driven by two promoters; and they give rise to multiple mRNA isoforms as a result of differential promoter activity, alternative splicing and the use of alternate transcription and polyadenylation signals.
To date five protein orthologs have been characterized that originate from the UT-A gene. UT-A mRNAs and proteins have been detected in several tissues including testis, vas deferens, and colon [1], however the tissue showing the greatest abundance, both in terms of the number of different isoforms present and level of expression, is the kidney.
In the mouse and rat kidney, isoforms UT-A1, UT-A2 and UT-A3 are the most abundant. UT-A1 is the largest UT-A isoform with 97 and 117kDa glycosylated protein forms. UT-A2 consists of the carboxyl terminal 397 amino acids of UT-A1 and exists as 43-55kDa glycosylated proteins. This isoform has been localized to type 1 and type 3 thin descending limbs (tDL) of the loop of Henle. In these nephron segments it is proposed to participate in intra-renal recycling of urea between the IMCD and loop of Henle. UT-A3 consists of the amino-terminal 460 amino acids of UT-A1 and occurs as 44 and 67 kDa glycosylated proteins. Both UT-A1 and UT-A3 localize to the inner medullary collecting duct (IMCD) of the kidney where they regulate urea reabsorption into the interstitium under the acute control of arginine vasopressin (AVP).
Through the creation of UT-A deficient mouse strains we and others have highlighted the central role of UT-A proteins in the urinary concentrating mechanism and this work has led to the reevaluation of several long standing hypotheses namely those advanced by Berliner [2], Kokko and Rector [3] and Stephenson [4]. Whereas mice devoid of UT-A2 show only a relatively mild defect in the urinary concentrating mechanism [5], mice lacking UT-A1 and UT-A3, have a severe concentrating defect. Interestingly, the severity of this phenotype is reciprocally related to the protein content of the diet [6]
To study the function and regulation of UT-A transporters at the cellular level, we have recently engineered MDCK type I cell lines to stably express mouse UT-A proteins. These cell lines, when grown on permeable supports, have enabled urea transporter function to be assessed via radiolabelled urea flux across a polarized epithelia cell monolayer. This approach has successfully been used to study acute regulation of UT-A1-, UT-A2- and UT-A3-mediated urea transport. In addition, we have also developed novel strategies to study urea transporter function. By making membrane vesicles from Xenopus oocytes expressing UT-A transporters and using light scattering techniques we have been able, for the first time, to measure the kinetics of UT-A transporter isoforms [7].
Finally, using mutagenesis and by making UT-A protein chimeras we are beginning to probe the structure of the UT-A proteins. This in combination with studies to optimize heterologously expression of bacterial and mammalian urea transporters should in the near future enable us to unlock the tertiary structure of these uniquely important molecules.

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

SA18

Epithelial calcium channels: uniquely regulated gatekeepers

J.G. Hoenderop

Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands

The calcium balance is tightly maintained by efficient feedback mechanisms involving parathyroid glands, bone, intestine and kidney. Changes in ionized calcium concentration are detected by the calcium-sensing receptor (CaSR) present in parathyroid cells, which subsequently modulates the parathyroid hormone (PTH) secretion. PTH in turn participates in the maintenance of plasma calcium levels within the physiological range by modulating bone resorption, renal calcium reabsorption and indirectly, by increasing the 1,25-dihydroxyvitamin D3 synthesis to regulate intestinal absorption of calcium. Importantly, genetic studies as well as molecular cloning strategies recently identified new epithelial ion channels as the gatekeepers of active calcium absorption processes. These channels are members of the transient receptor potential (TRP) superfamily. TRPV5 and TRPV6 are responsible for the rate-limiting calcium entry in kidney and intestine, respectively [2,3]. Dysregulation or malfunction of these influx pathways has been associated with renal wasting and intestinal malabsorption of calcium.

Ageing is tightly associated with a negative calcium balance leading to among others osteoporosis, arteriosclerosis and ectopic calcification. Recently, klotho was identified as the new 'anti-aging' hormone being instrumental in the process of age-related adaptations in the calcium balance. Klotho is a type I membrane glycoprotein which shares homology to β-glucosidase enzymes. Interestingly, klotho gene ablation resulted in a syndrome closely resembling human ageing, including short life span, bone aberrations, skin atrophy and a disturbed calcium balance together with high serum vitamin D levels. Recently, we showed that klotho is predominantly expressed in kidney, where it is secreted in serum and urine. Furthermore, we demonstrated that TRPV5 and TRPV6 are stimulated by klotho via a novel extracellular activation mechanism [1]. We demonstrated that klotho hydrolyses via its β-glucuronidase activity the extracellular TRP channel sugar residues, entrapping the channel protein in the plasma membrane to maintain durable calcium transport activity. Channel regulation by extracellular modification of the protein glycan is a novel activation mechanism and might be applicable to other membrane proteins as well.

The activity of TRPV5 and TRPV6 is controlled at the transcriptional and translational level by hormones and dietary content of divalents as studied in various animal models. Besides this long-term control, the epithelial channels can be regulated by trafficking to and from the plasma membrane and by direct activation at the plasma membrane as investigated in several cell models. The recent elucidation of channel-associated proteins has provided new molecular mechanisms underlying these processes. Our recently performed studies combined electrophysiological, biochemical, molecular biological and confocal fluorescent imaging techniques and should ultimately allow the establishment of a comprehensive cellular model of calcium (re)absorption.

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SA19

The regulation of epithelial Na^+ channels

D.I. Cook, L.M. O'Mullane, I. Lee and A. Dinudom

School of Medical Sciences, University of Sydney, Camperdown, NSW, Australia

Epithelial Na^+ channels play a critical role in the regulation of extracellular fluid volume and the thickness of the fluid layers that cover the mucosal surfaces of the respiratory tract and colon. The intracellular mechanisms that control their activity include inhibitory feedback by the intracellular concentrations of Na^+ and Cl^-, as well as protein kinases and phosphatases. Feedback regulation by intracellular Na^+ of the channels is mediated by the ubiquitin protein ligases, Nedd4 and Nedd4-2, which bind to proline-rich motifs (so-called PY motifs) in the carboxy termini of the beta and gamma subunits of the channel. The mechanism by which intracellular Cl^- regulates the channels remains unclear.

The kinases that regulate the channels include the G protein-coupled receptor kinase, Grk2, which activates the channels by phosphorylating them and rendering them refractory to the action of Nedd4 and Nedd4-2. Other kinases, such as the serum- and glucocorticoid-induced kinase, Sgk, activate the channels by mechanisms that do not affect Na^+ feedback regulation of the channels, and may not involve direct phosphorylation of the channel protein. Among the phosphatases that regulate the channels is protein phosphatase I, which counteracts the effects of Grk2 so as to increase the sensitivity of the channels to Nedd4 and Nedd4-2.

Epithelial Na^+ channels are also regulated by a wide variety of extracellular factors. These include hormones such as aldosterone, autocrine factors such as ATP and pathogens such as influenza virus and parainfluenza virus. The intracellular mechanisms by which these agents influence Na^+ channel activity are known in part. Aldosterone, for example, exerts its immediate actions by increasing expression of Sgk, while the effects of influenza virus are mediated by protein kinase C. The mechanisms by which ATP inhibits the channels are of particular interest since purinergic inhibition of epithelial Na^+ channels is of potential importance in the treatment of cystic fibrosis and because the inhibitory effects of parainfluenza virus on the channels are mediated by the autocrine action of ATP. ATP is known to act on purinergic receptors in both the the apical and basolateral membranes of epithelia and has been proposed to inhibit epithelial Na channels via increased intracellular Ca^{2+}, via protein kinase C, and via changes in the content of phosphoinositides in the cell membrane.

We have been using mouse mandibular salivary duct cells studied in the whole-cell patch-clamp configuration and Fischer Rat

Thyroid cells transfected with the α-, β- and γ-subunits of mouse ENaC and cultured on permeable supports to screen for novel intracellular regulators of the channels, and to identify the intracellular signalling pathways by which purinergic receptors regulate epithelial Na^+ channels. Our experiments have ruled out roles for protein kinase C and membrane phosphoinositides in mediating the actions of purinergic receptors on the channels, while indicating that phospholipase Cβ plays only a limited role. They also suggest that the role of intracellular Ca^{2+} in the purineric effects on the channels is mediated by changes in intracellular Cl^-.

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SA20

Regulation of TASK-2 channels – cellular and molecular aspects

M. Hunter

Institute of Membrane and Systems Biology, University of Leeds, Leeds, UK

TASK-2 is a member of the Tandem-pore domain Acid-Sensing K^+ channel (TASK) family. In common with the other TASK channel family members, it is sensitive to extracellular pH, and inhibited by acidity. TASK-2 is also sensitive to changes in the osmotic potential of the external medium and participates in cell volume regulation (Niemeyer et al. 2001). It is located in various epithelial tissues including the pancreas, placenta, lung, small intestine, colon and especially the kidney, yet is absent from excitable tissues such as the nervous system, skeletal muscle and the heart (Lesage & Lazdunski, 2000). TASK-2 deficient animals have a metabolic acidosis and hypotension due to the urinary loss of $NaHCO_3^-$ (Warth et al. 2004).

TASK-2 is thought to be located in the basolateral membranes of proximal tubule cells, where it participates in the reclamation of filtered HCO_3^- by maintenance of the basolateral membrane potential. Efflux of HCO_3^- across the basolateral membrane on the NBC cotransporter is thought to alkalinise the extracellular fluid in the restricted microenvironment surrounding the basolateral surface of the cells, raising the local pH and activating TASK-2.

We have investigated the regulation of TASK-2 using a mutational approach. Channel function was studied by transient expression of wild-type and mutant channels in CHO cells and subsequent whole-cell and single channel patch clamp.

The open probability of K2P channels is generally independent of voltage, yielding linear current/voltage (I/V) curves. Despite these properties, we found that these channels showed distinct inward rectification immediately upon the establishment of whole-cell clamp, which became progressively less pronounced with time. This rectification was unaffected by polyamines or Mg^{2+} (agents that cause rectification in Kir channels) but was mimicked by inclusion of Na^+, in the pipette solution (Morton et al. 2004). In excised inside-out patches, Na^+ reduced the amplitude of single channel currents, indicative of rapid block and unblock of the pore. Mutations in the selectivity filter abolished Na^+-induced rectification, suggesting that Na^+ binds within the selectivity filter in wild-type channels. This sensitivity to intracellular Na^+ may be an additional potential regulatory mechanism of TASK-2 channels.

The pH-sensing mechanism was different from that of TASK-1 and -3, where a single pore-neighbouring residue is largely responsible, involving the combined action of several charged residues in the large extracellular M1-P1 loop (Morton et al. 2005). Neutralisation of no single amino acid in isolation gave substantial loss of pH-sensitivity. However, the combined removal of five charged amino acids (E28, K32, K35, K42 & K47) resulted in a marked reduction in pH-sensitivity. Wild-type channels contain two M1-P1 loops, but a concatemeric construct, comprised of one wild-type subunit and one containing the five mutations, was fully pH sensitive, indicating that only one M1-P1 loop is required to yield a fully pH sensitive channel.

Unless prevented, channel activity in the whole cell mode decreases with time, such that roughly half of the channel activity is lost within 5 min. This run-down depends upon phosphorylation state and PKC activity via a mechanism involving the C-terminus, and may involve channel trafficking.

Thus the regulation of TASK-2 channels is multifactorial, as in most channels, and the prime determinant of channel activity may yet turn out to be something other than the extracellular pH.

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