C6

Phosphorylation of Kidney Anion Exchanger 1 (Band 3) on tyrosine residues Y359 and Y904 critical for basolateral trafficking

A.M. Toye, A.C. Brown, W.J. Mawby and R.C. Williamson

Biochemistry, University of Bristol, Bristol, UK

The human Anion exchanger 1 (AE1, band 3, SLC4A1) is highly expressed in erythrocytes and ∝-intercalated cells of the kidney. The kidney AE1 (kAE1) is a truncated version of AE1 found in the basolateral membrane and its correct localisation and function is essential for renal acid secretion. However, despite its importance in whole body pH homeostasis surprisingly little is known about the trafficking and regulation of human kAE1.

We have developed a polarisable cell model system expressing human kAE1 in MDCKI cells (MDCKI-kAE1 cells) to investigate the regulation of the trafficking of kAE1. We have already shown, using this model system, that the C-terminal tyrosine residue (Y904) is critical for basolateral localisation in polarised cells and that the N-terminus of kAE1 is also essential for polarized trafficking. We have now utilized mutagenesis to further investigate the regions of the N-terminus important for kAE1 trafficking. In particular, we have found that the Y359A mutant is mistargeted to the apical membrane. Therefore, kAE1 has a requirement for the presence of both Y359 in the N-terminus and Y904 in the C-terminus for correct trafficking.

Tyrosine phosphorylation can influence the localisation of proteins by regulating interactions with cellular targeting and/or internalisation machinery. Both Y359 and Y904 are phosphorylated in erythrocyte AE1. We show by cellular imaging and western blotting techniques using novel phosphotyrosine-specific antibodies raised against AE1 phosY359 and AE1 phosY904, that both Y359 and Y904 are phosphorylated in pervanadate treated MDCK-kAE1 cells. This phosphorylation was sensitive to Src kinase inhibitors. Therefore since both Y359 and Y904 are critical for basolateral targeting, we propose that reversible phosphorylation provides a means of regulating kAE1 trafficking to the basolateral membrane. We suggest that intercalated cells can regulate the levels and subcellular distribution of kAE1 in response to cellular stimuli and cues (such as acidosis or alkalosis) by reversible phosphorylation of Y359 and Y359. We are now exploring a range of stimuli to determine the physiological signals that influence kAE1 phosphorylation and their effects on basolateral localisation.

Funded by a NHS Blood and Transplant Wellcome Trust Career Development Fellowship and a Kidney Research UK and GSK PhD Studentship.

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

C7

Evidence against ClC-5 as an electrical shunt in endosomal acidification in the proximal tubule

J.D. Lippiat and A.J. Smith

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

ClC-5 is a member of the mammalian family of voltage-dependent Cl^- channels that acts as a Cl^-/H^+ exchanger (Picollo & Pusch, 2005; Scheel et al. 2005). Inherited mutations of *CLCN5* lead to defective albumin reabsorption in the proximal tubule (Dent's disease). At the cellular level, ClC-5 co-localises with v-ATPase in endosomes of the proximal tubule epithelial cells, where it is thought to provide a Cl^- conductance to counterbalance the active transport of H^+ into the endosomal lumen (Günther et al. 1998). Whether the physiological properties of ClC-5 permit this shunting role has yet to be fully addressed.

We transfected human embryonic kidney cells (HEK 293) with plasmid DNA encoding EYFP-tagged ClC-5 and recorded currents using the whole-cell patch clamp technique. The bath solution contained (in mM) 140 CsCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, and the pipette solution contained 42 CsCl, 98 aspartate, 10 EGTA, and 10 HEPES; both solutions adjusted to pH 7.4. Cells were held at -30mV and 10ms pulses were applied from -100 to +200mV. Cells expressing ClC-5-EYFP produced outwardly-rectifying currents with a half-maximal activation voltage of +110 ± 2mV and an equivalent gating charge of 1.32 ± 0.13 (mean ± s.e.m., n=5). These currents, when extrapolated to the endosomal membrane, correspond to a unidirectional flux of chloride from the lumen to the cytosol. Currents were measured with $[Cl^-]$ ranging from 10 to 140mM and pH from 8.4 to 5.4 on both sides of the membrane, but on no occasion were inward currents observed.

To further understand the physiological role of ClC-5 in endosomal membranes we studied acidification using a pH-sensitive fluorescent protein, pHluorin, targeted to endosomes by fusion to VAMP2 (Miesenbock et al., 1998). In an extracellular buffer with pH 7.4, untransfected HEK cells acidified endosomal contents to pH 6.98 ± 0.05 (n=25), but failed to acidify endosomes (pH 7.40 ± 0.08, n=15) in the presence of 25μM bafilomycin, a v-ATPase inhibitor. HEK cells expressing ClC-5 had endosomal pH 6.41 ± 0.18 (n=30) and in the presence of bafilomycin, pH 6.87 ± 0.18 (n=16), indicating a bafilomycin-insensitive acidification mechanism when ClC-5 is present.

The data suggest that, in endosomes, ClC-5 does not act as en electrical shunt, but conducts Cl^- ions from the lumen to the cytosol. Since this is coupled to an exchange for H+ this may provide an acidification mechanism that acts in parallel with vesicular v-ATPase. This enhanced acidification in early endocytic vesicles may be important for efficient albumin endocytosis in the proximal tubule.

Picollo A & Pusch M (2005). Nature 436, 420-423.

Scheel O, et al. (2005). Nature 436, 424-427.

Günther et al. (1998). PNAS 95, 8075-8080.

Miesenbock G et al. (1998). Nature 394, 192-195.

Supported by The Wellcome Trust. We thank Prof. R.V. Thakker for the ClC-5 clone.

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

C8

A range of functional consequences caused by Dent's disease missense mutations of ClC-5

A.J. Smith[1], A.A. Reed[2], N.Y. Loh[2], R.V. Thakker[2] and J.D. Lippiat[1]

[1]Institute of Membrane and Systems Biology, University of Leeds, Leeds, UK and [2]Academic Endocrine Unit, University of Oxford, Oxford, UK

ClC-5 is a member of the voltage-gated chloride channel family that acts as a Cl^-/H^+ antiporter [1,2]. It is expressed in the proximal tubule of the kidney where it involved in small-peptide reabsorption from the urine. ClC-5 is predominantly located on endosomal membranes and has been proposed to provide a Cl^- conductance to counterbalance the action of v-ATPase. Mutation of ClC-5 has been linked to Dent's disease, an X-linked disorder characterised by proteinurea, hypercalciurea and nephrolithiasis [3].

We have examined the functional consequences of several known Dent's disease missense mutations. Data are expressed as mean ± s.e.m., statistical significance determined by ANOVA. Following expression of wild-type (WT) and mutant EYFP-tagged ClC-5 in HEK293 cells, whole-cell currents were examined by patch-clamp electrophysiology. Current densities of cells expressing the G57V mutant (269±30pA/pF) were not different to WT (319±45pA/pF) ($p>0.05$, n=5) whereas R280P exhibited currents that were reduced by ~50% (155±46pA/pF) ($p<0.05$; n=5). Whole-cell currents of 5 other mutants (S270R, G513E, R516W, I524K & E527D) were not different to untransfected cells ($p<0.05$; n≥5). Confocal imaging of cells showed that WT, G57V, R280P and E527D were located predominantly in intracellular endosomes and to varying extents at the cell surface. The four remaining mutants were retained in the endoplasmic reticulum. Endocytosis was examined in cells expressing WT and endosome-targeted mutant ClC-5. The uptake of fluorescently-conjugated albumin was increased ~5-fold in cells expressing WT, G57V or R280P versus untransfected ($p<0.01$; n≥6) but not in E527D expressing cells ($p>0.05$; n=11). Uptake of fluorescently-conjugated dextran and transferrin were not affected by the presence of ClC-5 ($p>0.05$ vs untransfected; n≥4). Endosomal acidification was assayed using ratiometric pHluorin fused to VAMP2 for endosomal targeting [4]. Endosomes of untransfected cells bathed in pH 7.4 buffer were acidified to pH 6.98 ± 0.05. Significant acidification was observed in cells expressing WT (pH 6.40 ± 0.17), G57V (pH 6.25 ± 0.19) and R280P (pH 5.46 ± 0.15) ($p<0.01$ vs untransfected; n≥15 cells for each). Acidification in cells expressing E527D was impaired leading to a modest alkalisation compared to untransfected cells (pH 7.55 ± 0.09) ($p<0.05$; n=15 cells).

These data suggest that Dent's disease does not necessarily result from a loss of a ClC-5 conductance from the endosome, but could be caused by disruption of another unidentified process involving ClC-5. They also suggest that efficient albumin endocytosis requires functional ClC-5 at both surface and endosomal membranes.

Picollo A & Pusch M (2005). *Nature.* **436**. 420-423

Scheel O, *et al.* (2005). *Nature.* **436**. 424-427

Lloyd SE, *et al.* (1996). *Nature.* **379**. 445-449

Miesenbock G, *et al.* (1998). *Nature.* **394**. 192-195

Supported by the Wellcome Trust

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

C9

Role of WT1 in the regulation of expression of pro- and anti-angiogenic isoforms of VEGF

E. Amin[1,2], D.G. Nowak[2,1], S.J. Harper[2], D.O. Bates[2] and M.R. Ladomery[1]

[1]Centre for Research in Biomedicine, University of the West of England, Bristol, UK and [2]Microvascular Research Laboratories, University of Bristol, Bristol, UK

The Wilms tumour suppressor gene WT1 is mutated in 10-15% of Wilms tumours and mutations in WT1 are also associated with Denys Drash Syndrome (DDS). DDS is characterised by childhood nephrotic syndrome, glomerulosclerosis, end-stage renal failure and Wilms tumours. WT1 encodes a zinc finger transcription factor that also regulates gene expression at the mRNA level. The search for WT1's DNA and mRNA targets is ongoing – a recent report has suggested that WT1 regulates the expression of a key growth factor, VEGF (Vascular Endothelial Growth Factor). There are two families of VEGF isoforms; an angiogenic family ($VEGF_{xxx}$); and a sister family of anti-angiogenic isoforms ($VEGF_{xxx}b$) that result from a distal alternative 3' splice site in exon 8 (Bates et al 2002). Although WT1 regulates VEGF expression transcriptionally, the extent to which WT1 affects VEGF isoform levels is not known, but splicing is altered in Denys Drash patients (Schumacher et al 2007). We measured VEGF isoform expression in differentiated wild-type and DDS patient-derived podocytes with a mutation in WT1 (R366C). The differentiated podocytes represents the closest in vivo model of podocytes in the kidney within the constraints of a cell culture model. Total VEGF and $VEGF_{xxx}b$ levels were determined by ELISA and RT-PCR. In the differentiated DDS podocytes there was a 0.13 ±0.002 fold reduction in the amount of $VEGF_{xxx}b$ compared to wildtype differentiated podocytes while DDS stably transfected with wildtype WT1 restored the expression of $VEGF_{xxx}b$ to 0.73 ±0.08. In the differentiated DDS podocytes there was a 2.32 ±0.483 fold increase in the amount of panVEGF compared to wildtype differentiated podocytes. Transfection of the wildtype WT1 into the differentiated DDS podocytes manages to rescue the DDS phenotype by decreasing the amount of panVEGF to 0.618 ±0.114 fold compare to wildtype podocytes. These results suggest that WT1 plays a role in the regulation of VEGF isoform expression; either by direct binding to VEGF pre-mRNA or by modulating splice factor activities

C13

Angiopoietin-1 increases albumin reflection coefficient of isolated mouse glomeruli *ex vivo*

L. Sage, C.R. Neal, J.K. Ferguson, S.J. Harper, D.O. Bates and A.H. Salmon

Physiology, University of Bristol, Bristol, UK

Albuminuria represents excessive macromolecular permeability of the glomerular filtration barrier (GFB) and is a common feature of kidney disease. Angiopoietin-1 (Ang1) is the only podocyte-produced molecule shown to reduce the reflection coefficient (σ) for albumin (σ_{alb}) of systemic microvessels[1]. We hypothesised that Ang1 would decrease glomerular albumin permeability.

We previously described a refinement of a technique to measure water permeability (L_pA) in single isolated glomeruli, in which an oncotic pressure gradient drives fluid flux across the GFB, resulting in changes in glomerular volume[2]. We have adapted this technique to measure glomerular σ_{alb}[3]. σ describes the fraction of molecules retained by a membrane: for very large molecules that are perfectly retained by the GFB (e.g. >100kDa dextran), $\sigma = 1$; for albumin, σ of the GFB is just less than 1. A solution of albumin molecules will therefore exert a lower oncotic pressure across the GFB than an equivalent solution of dextran molecules. The ratio of these effective oncotic pressures is used to calculate σ_{alb}.

250kDa dextran was dialysed across a 100kDa membrane to eliminate smaller fragments that can cross the GFB. We created BSA and dextran solutions iso-oncotic to mouse plasma [π=32.2cm H_2O][4]. Kidneys were extracted from humanely sacrificed Tie-2GFP mice. Glomeruli were isolated by flushing renal cortical tissue through graded metal sieves. Glomeruli were aspirated onto a micropipette within a flow-controlled observation chamber, then perifused with BSA and examined under static conditions for 2min. Perifusate was then switched to dextran. Glomerular volume during each perifusate incubation period (dextran: V_{dex}; albumin: V_{alb}) was recorded on video, then calculated using ImageJ and Adobe Photoshop software. The ratio V_{dex}/V_{alb} describes σ_{alb}.

Paired measurements of σ_{alb} were obtained in glomeruli before and after exposure to either 200ng/ml Ang1 or control solution for 1 hour. Whereas treatment of glomeruli with control solution did not alter σ_{alb} [from 0.9608 to 0.9639, p>0.35, paired t test, n=15], treatment with Ang1 significantly increased σ_{alb} [from 0.9725 to 0.9804, p<0.05, paired t test, n=15]. These results indicate a reduction in the macromolecular permeability of the GFB after treatment with Ang1 and support further research with Ang1 as a potential novel therapy in kidney disease.

Salmon AH *et al.* (2006). *Microcirc* **13(6)**, 531.

Salmon AH *et al.* (2006). *J Physiol* **570**, 141-56.

Sage LM *et al.* (2007). *Microcirc* **14(6)**, 653.

Stohrer M *et al.* (2000). *Cancer Res* **60(15)**, 4251-5.

Supported by Kidney Research UK.

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

C14

TRPC6 acts as a unique store calcium-release channel in podocytes, in a nephrin-dependent manner

R.R. Foster[1], M.A. Zadeh[2], G.I. Welsh[1], S.C. Satchell[1], P.W. Mathieson[1], D.O. Bates[2] and M.A. Saleem[1]

[1]Clinical Science @North Bristol, University of Bristol, Bristol, UK and [2]Microvascular Research Laboratories, University of Bristol, Bristol, UK

Mutations in TRPC6 lead to increased intracellular calcium ($[Ca^{2+}]_i$) signalling and a phenotype of familial nephrotic syndrome, affecting the glomerular podocyte (1,2). In addition, in some acquired glomerulopathies increased podocyte TRPC6 expression is associated with the re-localisation, or downregulation of nephrin (3,4,5,6), a cell adhesion molecule and podocyte marker associated with the slit diaphragm. We sought to determine how podocytes are uniquely affected by TRPC6 mis-function and the role of nephrin using human conditionally immortalised podocytes (ciPods) and nephrin-deficient (ND) ciPods, previously characterised (7,8).

Cells were loaded with Fura2-AM, Methyl β cyclodextrin (MβCD), when used was added in the final 30mins of loading. Cells, incubated in Krebs' solution containing 1.5mM or 200nM Ca^{2+}, were stimulated with 200μM flufenamic acid (FFA) in the presence or absence of thapsigargin (TG). $[Ca^{2+}]_i$ was proportional to the fluorescence intensity ratio (R_{norm}).

ciPods transfected with Ad-virus containing wild type and dominant negative TRPC6 constructs verified the specificity of FFA to TRPC6. 200μM FFA increased the R_{norm} in ciPods in 1.5mM $[Ca^{2+}]_o$ (1.44±0.11 fold increase, p≤0.01 paired t-test) and surprisingly in 200nM $[Ca^{2+}]_o$ (1.58±0.01 fold increase, P≤0.01, paired t-test), but not after store-depletion (R_{norm}1; 1.22±0.06 fold increase) in contrast to an increase in ND ciPods after store-depletion (1.6±0.06 fold increase, p≤0.05, paired t-test). Pre-incubation of ciPods with 1mM MβCD inhibited the response to FFA in 1.5mM $[Ca^{2+}]_o$ as expected (R_{norm} 1.07±0.03 fold increase), yet also blocked the response in 200nM $[Ca^{2+}]_o$ (R_{norm}1.19±0.01 fold increase), verifying TRPC6 function as a store calcium release channel.

In conclusion, FFA specifically activates TRPC6 in ciPods. In these cells TRPC6 can uniquely act as a store calcium-release channel, although only in the presence of nephrin. The activation of TRPC6 is lipid raft dependent, whether in the plasma membrane or on internal stores.

Winn MP et al. (2005). Science 308:1801-1804.

Reiser J et al. (2005). Nat Genet 37:739-744.

Moller CC et al. (2007). J Am Soc Nephrol 18:29-36.

Doublier S et al. (2001). Am J Pathol 158:1723-1731.

Kim BK et al. (2002). Am J Kidney Dis 40:964-973.

Coward RJ et al. (2005 J Am Soc Nephrol 16:629-637.

Saleem MA et al. (2002). J Am Soc Nephrol 13:630-638.

Foster RR et al. (2005). Am J Physiol Renal Physiol 288:F48-57.

This project was funded by the MRC.

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

C15

The effect of hypertension on the filtration barrier of subpodocyte spaces in rat glomerular capillaries

C.R. Neal, V.J. Murdin, P.R. Muston, D.O. Bates and S.J. Harper

Microvascular Research Labs, Physiology Dept, University of Bristol, Bristol, UK

Bowman's space consists of three interconnected urinary spaces forming drainage pathways for ultrafiltrate (Neal CR *et al.* 2005, 2007). Glomerular filtrate may enter restrictive urinary spaces under the podocyte cell body (subpodocyte space, SPS) before passing to other urinary spaces. We have measured SPS in 12 week old (early) spontaneously hypertensive rats (SHR) or Wistar Kyoto rats (WKY) controls after fixation at physiological oncotic and hydrostatic pressures.

SHR (mean arterial pressure (MAP) 140mmHg) or WKY (MAP 96mmHg) kidneys from pentobarbital (i.p.) euthanased rats were perfusion fixed at matched MAP with isoncotic glutaraldehyde solutions. Serial ultrathin sections of kidney were used to reconstruct regions of glomeruli. From these filtration slit density of the glomerular capillary wall, SPS coverage of the capillaries, and the available area for filtration into the SPS was measured and compared (mean±SEM, unpaired t-test).

The filtration slit density along the glomerular filtration barrier in non-SPS regions of the SHR and WKY was the same (2.3±0.2 per μm, n=14, 2.3±0.1 per μm, n=15). However, filtration slit density decreased in the covered SPS regions of SHR glomerular capillaries (1.8±0.1per μm, n=14, $P<0.05$) compared with WKY controls (2.1±0.1per μm, n=15). The SPS covered area of the glomerular filtration barrier was similar in both (62±14% to 72±9%).

Mild hypertension in 12 week old SHR produces a decrease in the number of filtration slits of the filtration barrier covered by SPS. This reduction in filtration pores suggests a lower permeability in these SPS covered regions compared to uncovered regions (non-SPS).

Neal CR *et al.* (2005). *J Am Soc Nephrol* 16, 1223-35.

Neal CR *et al.* (2007). *Am J Physiol Renal Physiol* Sep 5; (Epub ahead of print).

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

C16

Podocyte specific over-expression of VEGF-A_{165}b significantly reduced the normalised ultrafiltration coefficient in the absence of any change in reflection coefficient

J.K. Ferguson, Y. Qiu, L.M. Sage, C.R. Neal, D.O. Bates, S.J. Harper and A.H. Salmon

Physiology (MVRL), University of Bristol, Bristol, UK

The glomerular filtration barrier (GFB) has three main cellular components: fenestrated endothelial cells; basement membrane; and podocyte foot processes. In health the GFB is highly permeable to water and small solutes but almost entirely impermeable to proteins. Vascular endothelial growth factor A (VEGF-A) is produced by podocytes. Tight regulation of VEGF-A expression is critical for the establishment and maintenance of the GFB[1], and glomerular VEGF expression is disrupted in many glomerular diseases[2]. Differential splicing of the VEGF-A gene forms two families of isoforms: the pro-angiogenic family (VEGF-A_{xxx}), and the anti-angiogenic families (VEGF-A_{xxx}b). VEGF-A_{165} contributes to the high permeability of glomeruli to water[3]. We sought to determine the effects of VEGF-A_{165}b on glomerular permeability by examining permeability coefficients in glomeruli harvested from transgenic mice with podocyte specific heterozygosity ($^{+/-}$) for VEGF-A_{165}b.

The water permeability of isolated mouse glomeruli (normalised ultrafiltration co-efficient: L_pA/V_i) was assessed using a previously described oncometric technique[3]. L_pA/V_i is calculated from the rate of change of glomerular volume after introduction of an oncotic pressure gradient of known magnitude. VEGF-A_{165}b over-expression reduced glomerular L_pA/V_i as compared with wild-type littermate controls (VEGF-$A_{165}b^{+/-}$: 1.44±0.11, n=18; controls: 1.93± 0.16, n=8; unpaired t-test: p=0.017).

We have modified this methodology to estimate the reflection coefficient to albumin (σ_{alb})[4]. The volume of a glomerulus incubated in dextran solution, expressed as a fraction of the volume of the same glomerulus when incubated in an iso-oncotic solution of albumin, describes σ_{alb}. Compared with wild-type littermate controls, there was no significant difference in the σ_{alb} in glomeruli harvested from VEGF-$A_{165}b^{+/-}$ mice (VEGF-$A_{165}b^{+/-}$: 0.975±0.011, n=9; controls: 0.998± 0.02, n=11; unpaired t-test p=0.32).

These results suggest that a higher concentration of podocyte VEGF-A_{165}b does not reduce the ability of the barrier to retain macromolecules. Altering the balance of VEGF-A_{xxx} and VEGF-A_{xxx}b in disease states may be of therapeutic interest.

Eremina V *et al.* (2003). *J Clin Invest* **111**, 707-716.

Schrijvers KI *et al.* (2004). *Kidney Int* **65** (**6**), 2003-2017.

Salmon AH *et al.* (2006). *J Physiol* **570**, 141-56.

Sage LM *et al.* (2007). *BMS Spring Meeting, Belfast.*

Supported by the British Heart Foundation.

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

PC1

The revertant mutants G550E and 4RK alter the processing efficiency and gating behaviour of G551D-CFTR

Z. Xu[1], L.S. Pissarra[2,3], C.M. Farinha[2,3], D.N. Sheppard[1] and M.D. Amaral[2,3]

[1]Dept. Physiol. & Pharm., University of Bristol, Bristol, UK, [2]Dept. Chem. & Biochem., University of Lisboa, Lisboa, Portugal and [3]Centre of Human Genetics, National Institute of Health, Lisboa, Portugal

The simultaneous mutations of four arginine-framed tripeptides (AFTs) (R29K, R516K, R555K and R766K; 4RK) in cystic fibrosis transmembrane conductance regulator (CFTR) rescues the processing and function of the most common cystic fibrosis mutation, F508del [1]. G550E, another F508del revertant, exerts, by itself, a similar effect [2]. Recently, we demonstrated that 4RK affects mainly processing efficiency, whereas G550E appears to act directly on CFTR structure, as it rescues efficaciously F508del gating [3]. Of note, the G550E revertant is located next to G551, the site of a CF mutant (G551D) that profoundly disrupts CFTR gating. Here, we explore the effects of G550E and 4RK on the processing and gating of G551D. We used BHK cells expressing G551D-, G551D-G550E- and G551D-4RK-CFTRs, metabolic pulse-chase labelling to study protein processing and excised inside-out membrane patches to examine channel gating [3]. Data are means ± SEM of n observations; statistical analyses were performed using Student's paired t test.

Unlike F508del, G551D is processed normally. However, the processing efficiency of G551D was modulated by revertant mutants with G550E, but not 4RK, increasing processing efficiency. These data contrast with the effects of the revertants on wild-type (wt) and V562I, where 4RK, but not G550E, enhanced processing efficiency [3]. In contrast to wt, the interburst interval (IBI) of G551D was prolonged greatly and the mean burst duration (MBD) reduced with the result that P_o was diminished markedly (wt, P_o = 0.47±0.03, n = 7; G551D, P_o = 0.007±0.001, n = 8). Although both G550E and 4RK increased the P_o of G551D, they were ineffective at rescuing channel activity. Surprisingly, while both revertants attenuated markedly IBI, they also shortened MBD (G551D-G550E, P_o = 0.010±0.001, MBD = 27±4 ms, IBI = 2,772±301 ms, n = 13 for all; G551D-4RK, P_o = 0.027±0.005, MBD = 11±1 ms, IBI = 535±113 ms, n = 14 for all; $p < 0.05$). As a result, the gating behaviour of G551D-G550E and G551D-4RK appeared very flickery.

To summarise, the revertant G550E increased the processing of G551D, but not that of wt and V562I [3], whereas, both G550E and 4RK restored function to F508del [3], but not G551D. Our data suggest that when in cis with G551D, AFTs might influence directly channel gating, arguing that 4RK, in addition to its effects on trafficking, might alter G551D folding. Thus, the effects of G550E and 4RK on protein processing and channel gating are mutation-specific.

Chang X-B et al. (1999). Molecular Cell 4, 137-142.

DeCarvalho AC et al. (2002). J Biol Chem 277, 35896-35905.

Roxo-Rosa M et al. (2006). Proc Natl Acad Sci USA 103, 17891-17896.

Supported by CF Trust (UK) and FCT (Portugal). ZX is supported by an ORS award and a University of Bristol Scholarship.

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

PC2

Effects of the KCNQ1 K^+ channel inhibitor chromanol 293B on renal function in wildtype and KCNE1 knockout mice

A. Neal[1], J. Kibble[3], S. White[2] and L. Robson[1]

[1]Biomedical Science, University of Sheffield, Sheffield, South Yorkshire, UK, [2]Institute of Membrane and Systems Biology, University of Leeds, Leeds, West Yorkshire, UK and [3]Medicine, Memorial University Newfoundland, St. John's, NF, Canada

The K^+ channel β subunit KCNE1 plays a role in proximal tubule function, maintaining electrogenic transport. KCNE1 knockout (KO) mice show increased urinary excretion of Na^+, glucose and fluid (Vallon, 2001). KCNE1 regulates KCNQ1, which is found in the kidney. However, the renal response in KCNQ1 KO mice is different (Vallon, 2005). In addition, a recent report indicated a distal location for KCNQ1 (Zheng, 2007). The aim of this study was to examine the renal response to chromanol 293B, a KCNQ1 inhibitor, comparing responses in wildtype (WT) and KCNE1 KO mice. Adult WT and KCNE1 KO mice were anaesthetised initially with Na^+ thiopentone (100 mg/kg) and anaesthesia maintained with ketamine (10mg/kg) and xylazine (1.5 mg/kg), all intra peritoneal injections. They were then surgically prepared for renal clearance measurements. Animals received an intravenous infusion of 2.25g/dl BSA in isotonic saline. After surgery, chromanol 293B (12 μmol/kg/hr) or clofilium (K^+ channel inhibitor, 9 μmol/kg/hr) were administered. After 45 minutes equilibration urine was collected over 60 minutes. 3H-inulin was infused to allow determination of GFR. Controls received an equivalent dose of vehicle (DMSO). At the end of the collection period, a terminal blood sample was taken to obtain plasma. Statistical significance was assessed using ANOVAs and treatment tests, with significance assumed at the 5% level. Knockout of KCNE1 increased urine flow and the fractional excretion (FE) of Na^+ and Cl^- (Table 1). The FEglucose was unaffected in KO animals, 0.30 ± 0.06% versus 0.26 ± 0.06% in WT and KO, respectively. Infusion of chromanol 293B in WT mice mimicked the response observed in KO animals, while chromanol was without effect in KO mice. In contrast, clofilium increased the FE of Na^+ and Cl^- in WT and KO animals. There was no effect on urine flow, probably due to a reduced GFR in the clofilium treated mice. These data indicate that KCNE1 regulates a chromanol 293B sensitive K^+ channel in renal tubules. Given the previous work on KCNE1, the location of this channel is likely to be the proximal tubule, although further work is needed to confirm both channel location and identity.

Table 1: Renal parameters in WT and KO mice

between the PT and other nephron segments, which may prove to be important in the nature and localization of renal cell injury.

1. Martin-Hernandez E et al. (2005). Pediatr Nephrol 20:1299-1305.

2. Sipos A et al. (2007). Kidney Int Aug 1.

3. Duchen MR et al. (2003). Methods Enzymol 361, 353-89.

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

PC6

Use of REFER analysis to map the gating pathway of the human CFTR Cl^- channel

Z. Cai and D.N. Sheppard

Department of Physiology and Pharmacology, Bristol University, Bristol, UK

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel gated by ATP-driven nucleotide-binding domain (NBD) dimerisation. To investigate the gating pathway of CFTR, we performed rate-equilibrium free energy relationships (REFER) measurements (1, 2). We used site-directed mutations as structural probes, and ATP and voltage as environmental probes of channel gating (1) and C1↔C2↔O scheme to describe CFTR channel gating (3). C1 represents the long duration closed state separating channel openings and C2↔O the bursting state. Transitions between C1↔C2 and C2↔O are described by the forward rate constants β1 and β2 and the backward rate constants α1 and α2, respectively. As CFTR is gated by intracellular ATP, C1↔C2 reflects agonist binding step, whereas C2↔O reflects the gating step (4). REFER analysis is applied to the gating step. β2 and α2 are used to generate a Brønsted plot (log (β2) plotted vs. log (β2/α2)). The slope of the line in a Brønsted plot (Φ) quantifies the relative extent to which the opening (β2) and closing (α2) rate constants change and provides an estimate of the temporal sequence of intermediate events during channel gating. Φ ranges between 0 and 1. When Φ is close to 1, the transition-state resembles an open-channel conformation and moves early during gating, whereas when Φ is close to 0, the transition-state resembles a closed-channel conformation and moves late during gating (1, 2).

Brønsted plots of mutations (G550E, G551D, V562I, G551D-G550E, V562I-G550E) in the H5 α-helix of NBD1 (n = 3–14), different ATP concentrations (0.03–5 mM, n = 8–33) and a membrane voltage series (-100 – +100 mV, n = 5–7) yielded Φ values of 0.64 (r^2 = 0.91), 0.84 (r^2 = 0.55) and 0.18 (r^2 = 0.71), respectively. Good linear fits of the Brønsted plots of these perturbations indicate that CFTR is amenable to REFER analysis. We interpret our data to suggest that at the transition state, i) the structure of the H5 α-helix is a hybrid, which is more open than closed; ii) the ATP-binding sites are almost completely in the open state, arguing that the conformations of the ATP-binding sites change early in the CFTR gating pathway; iii) the CFTR pore is almost closed at the transition state, suggesting that the conformation of the CFTR pore changes late in the CFTR gating pathway. We conclude that there is a spatial gradient of Φ values from ~0.90 at the ATP-binding sites to ~0.20 at the CFTR pore. Thus, as proposed by Grosman *et al* (1), initiation at the effector site and propagation to the active site is likely to be a common theme for the gating of ion channels activated by ligands.

Grosman C *et al.* (2000). *Nature* 403, 773-776.

Purohit P *et al.* (2007). *Nature* 446, 930-933.

Winter MC *et al.* (1994). *Biophys J* 66, 1398-1403.

Colquhoun D (1998). *Br J Pharmacol* 125, 923-947.

We thank LS Pissarra and MD Amaral for the generous gift of CFTR constructs and Z Xu for excellent assistance. This work was supported by the BBSRC and CF Trust.

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

PC7

Membrane depolarisation indirectly regulates the Ca^{2+}-activated Cl^- conductance in mouse renal inner medullary collecting duct cells (mIMCD-3)

S.H. Boese[1], J.E. Linley[3], M.A. Gray[2] and N.L. Simmons[2]

[1]Zoophysiology, University of Potsdam, Potsdam, Germany, [2]Institute for Cell & Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK and [3]Institute of Membrane & Systems Biology, University of Leeds, Leeds, UK

Renal inner medullary collecting duct cells (mIMCD-3) possess a Cl^- conductance (CaCC) regulated by intracellular Ca^{2+} via Ca^{2+} influx across the apical plasma membrane (Stewart et al, 2001). Tpc1 is a Ca^{2+} permeable channel activated by depolarisation which is expressed in mIMCD-3 cells (Sayer et al, 2006) but whose physiological role is undefined.

Here we investigate the effect of membrane depolarisation on CaCC in mIMCD-3 cells. Membrane conductance of cells on coverslips was measured using the slow whole cell patch-clamp technique. Data are given as mean ± SEM (n), difference between mean values was determined by Student's t test.

When the membrane potential was held at -60mV (V_{hold}) with brief excursions to +60mV, only a small basal K^+ conductance was detected. In contrast, when V_{hold} was 0mV with brief excursions to ±60mV, there was a slow increase in conductance following a delay of ~60s. Whole cell conductance increased from 30.7 ± 3 & -18.4 ± 2pA/pF (at V_{hold} = -60mV) to 214.4 ± 22 & -108.9 ± 9pA/pF (at V_{hold} = 0mV) (n = 37) reaching a plateau after ~15mins ($T_{0.5}$ = 472 ± 23s). Currents were outwardly-rectifying, time-independent and Cl^- selective. Repolarisation (V_{hold} = –60mV), reversed the conductance increase to control values ($T_{0.5}$ = 90 ± 10s, n=15).

The sensitivity to depolarisation and dependence on Ca^{2+} influx suggests the involvement of a tpc1 like channel in CaCC activation. A distinguishing feature of tpc1 is its Al^{3+} sensitivity (Kawano et al, 2004). Whereas 1mM Al^{3+} did not effect basal conductance at –60mV, the activation of CaCC was abolished by 1mM Al^{3+} when V_{hold} was 0mV. The effect of 1mM Al^{3+} was com-

passage of labelled albumin by 30% relative to controls (n=10, p<0.001).
Conclusions:
High glucose *milieu* reduces the expression of sugar chains of glycocalyx without any alterations in their core proteins. This leads to a selective increase flux of albumin across GEnC monolayers without affecting the passage of water and solutes. These results support the role of GEnC glycocalyx in the pathophysiology of proteinuria in diabetes.

A.S. has received a training fellowship from Kidney Research UK.

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

PC12

Cation channel activity of Band 3 (AE1) mutations in distal renal tubular acidosis

S.B. Walsh[1,2], H. Guizouarn[2], F. Borgese[2] and R.J. Unwin[1]

[1]Department of Physiology and Centre for Nephrology, University College London, London, UK and [2]Bâtiment de Sciences Naturelles, Université de Nice-Sophia Antipolis, Nice, France

Hypokalemia is a common and incompletely explained finding in distal renal tubular acidosis (dRTA); its severity depending on the cause. Hereditary dRTA can be due to mutations in the anion exchanger 1 (AE1, Band 3). Recent work has shown that AE1 mutations associated with hereditary stomatocytosis (HSt) - an erythrocyte defect that can be a cause of pseudohyperkalemia - can behave as non-selective cation channels[1].
To investigate whether dRTA-causing AE1 mutants could also contribute to renal potassium wasting in dRTA via a similar mechanism, we studied 3 European autosomal dominant dRTA-causing mutants (R589H, G609R, S613F) and one Thai autosomal recessive mutant (G701D); each co-expressed with glycophorin A (GPA - to enhance plasma membrane expression) in *Xenopus Laevis* oocytes.
Chloride influx experiments showed that anion transport activity is preserved and similar to wild type (wt), in contrast to the cation leaky HSt mutants described previously. However, rubidium (Rb^+) influx experiments showed significantly higher cation flux in the western mutants compared with wt. Moreover, G701D had a very large cation flux, which was greatest at 0ºC and was inhibited by stilbene AE1 inhibitors. These results were confirmed by measurement of intracellular cations: mutant-expressing oocytes, notably G701D, had reversal of intracellular cation concentrations compared with wt. In addition, when G701D was co-expressed with wt AE1 in the absence of GPA, a strong Rb^+ flux was still found, indicating stability of the cation channel with heterodimer formation.
The G701D mutation is particularly frequent in NE Thailand[2], where dRTA and severe hypokalemia are endemic[3], and hypokalemic paralysis, and sudden death, are unusually common[4,5]. The novel behaviour of the AE1 mutants we have observed may contribute to potassium wasting in G701D homo and heterozygotes and thus add to the burden of hypokalemic disease seen in NE Thailand.

Bruce, L.J. *et al.* (2005) *Nature Genetics* **37**, 1258-1263.

Yenchitsomanus, P. T.*et al.* (2003) *Journal of human genetics* **48**, 451-456.

Nilwarangkur, S.*et al.* (1990) *The Quarterly journal of medicine* **74**, 289-301

Nimmannit, S.*et al.* (1991) *Lancet* **338**, 930-932.

Nademanee, K.*et al.* (1997) *Circulation* **96**, 2595-2600.

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

PC13

P2 receptors in mouse cortical collecting duct revisited

E. Odgaard, H.A. Praetorius and J. Leipziger

Physiology and Biophysics, Aarhus University, Aarhus, Denmark

P2 receptors are functionally expressed along the distal renal tubule and their activation regulates Na+, K+ and H2O transport1,2. Strong evidence highlights a crucial role of the P2Y2 receptor in this segment. Activation of luminal (and basolateral) P2Y2 receptors inhibit ENaC-mediated Na+ absorption3 and global KO of this receptor causes augmented renal Na+ and H2O absorption and increased systemic blood pressure 4. In this study we used the isolated perfused tubule technique together with fluo-4 intracellular Ca2+ imaging in P2Y2 WT and KO mice cortical collecting ducts to more comprehensively search for functionally relevant expression of luminal and basolateral P2 receptors. We find/confirm that luminal and basolateral ATP/UTP applied from either sides of the epithelium are potent agonists to trigger [Ca2+]i increases. (WT % fluo-4 increase, luminal UTP: 56±9 and luminal ATP: 47±10; basolateral UTP: 32±7 and basolateral ATP: 26±4, n=5). In KO tubules the luminal and basolateral UTP effect were absent (KO % fluo-4 increase, luminal UTP: 3±1; basolateral UTP: 0±0, n=14). Intriguingly, despite the ineffectiveness of luminal UTP in P2Y2 KO tubules, luminal ATP continued to trigger [Ca2+]i increases, which amounted to about 50% of the WT luminal ATP response (KO % fluo-4 increase, luminal ATP: 19±5) . Basolateral ATP-triggered [Ca2+]i responses were reduced in KO tubules (KO % fluo-4 increase, ATP: 16±2). These results define that mouse cortical collecting ducts express luminal and basolateral P2Y2 receptors. In addition, yet another luminal P2 receptor is present in this segment. Based on literature a P2X type receptor is suggested. The basolateral membrane is likewise equipped with at least one other P2 receptor, its nature remains to be established. These results extend the current state of knowledge of these receptors in the collecting duct and demand further in depth investigation of their functional relevance in salt and water handling.

1. Leipziger J (2003). Am J Physiol Renal Physiol 284, F419-F432.

2. Unwin RJ, Bailey MA & Burnstock G (2003). News Physiol Sci 18, 237-241.

3. Lehrmann H, Thomas J, Kim SJ, Jacobi C & Leipziger J (2002). J Am Soc Nephrol 13, 10-18.

4. Rieg T, Bundey RA, Chen Y, Deschenes G, Junger WG, Insel PA & Vallon V (2007). Mice lacking P2Y2 receptors have salt-insensitive hypertension and facilitated renal Na+ and water excretion. June, e-pub ahead.

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

PC14

Inhibition of the CFTR Cl^- channel by loop diuretics

M. Ju, T.S. Scott-Ward, Z. Cai and D.N. Sheppard

Department of Physiology and Pharmacology, University of Bristol, Bristol, UK

Loop diuretics are widely used to inhibit the Na^+-K^+-2Cl^- cotransporter (Haas & Forbush, 2000). However, Venglarik (1997) demonstrated that loop-diuretics inhibit the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. To understand better how loop diuretics inhibit CFTR, we studied furosemide, bumetanide, and two other agents xipamide and piretanide, which are structurally related to furosemide and bumetanide, respectively. We recorded CFTR Cl^- currents in inside-out membrane patches excised from C127 cells expressing wild-type human CFTR. The pipette (external) solution contained 10 mM Cl^- and the bath (internal) solution contained 147 mM Cl^-, 0.3 mM ATP and 75 nM PKA at 37°C; voltage was –50 mV. Data are means ± SEM of n observations and statistical analyses were performed using Student's paired t test.

When added to the internal solution, loop diuretics caused a reversible, concentration-dependent decrease in CFTR Cl^- current. For all agents tested, the concentration-response relationship was well fitted by the Hill equation with Hill coefficients of ~1. The rank order of potency for CFTR inhibition was xipamide (K_i = 45 ± 4 μM) ≥ bumetanide (K_i = 56 ± 11 μM) = piretanide (K_i = 58 ± 18 μM) ≥ furosemide (K_i = 71 ± 15 μM) (n = 5 for all agents).

To investigate further channel block, we used noise analysis. In the absence of furosemide, power density spectra of CFTR Cl^- currents were best fitted with two Lorentzian components with corner frequencies f_{c1} and f_{c2} of 1.17 ± 0.6 and 81 ± 10 Hz (n = 4), whereas in the presence of furosemide (100 μM), power density spectra were best fitted with three Lorentzian components with f_{c1}, f_{c2} and f_{c3} of 2.64 ± 1.38, 63 ± 25 and 312 ± 107 Hz (n = 4), respectively, suggesting that f_{c3} corresponds to the rapid binding and dissociation of furosemide to and from individual CFTR Cl^- channels. Consistent with this idea, furosemide (100 μM) caused a flickery block of CFTR decreasing both open probability (P_o: control, 0.41 ± 0.03; furosemide (100 μM), 0.17 ± 0.02; n = 6; $p < 0.01$) and single-channel current amplitude (i: control, –0.76 ± 0.01 pA; furosemide (100 μM), –0.63 ± 0.03 pA; n = 6; $p < 0.01$). Thus, our data demonstrate that loop diuretics inhibit CFTR, their potency approaches that of the widely used CFTR blocker glibenclamide and that furosemide acts an open channel blocker of CFTR.

Haas M & Forbush III B (2000). *Ann Rev Physiol* **62**, 515-534.

Venglarik CJ (1997). *Pediatr Pulmonol Suppl* **14**, 230.

We thank Dishman Pharmaceuticals & Chemicals Ltd and Sanofi-Aventis for generous gifts of xipamide and piretanide, respectively. This work was supported by the CF Trust.

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

PC15

Anion transport across planar lipid membranes by an artificial anionophore

G. Magro[1,2], L.W. Judd[2], D.N. Sheppard[1] and A.P. Davis[2]

[1]Physiology, University of Bristol, Bristol, UK and [2]Chemistry, University of Bristol, Bristol, UK

Natural and artificial ion transporters consisting of transmembrane channels or carriers are well known. Most of the synthetic transporters concern cation transport, whereas less interest has been shown in anion transport so far. In previous work (Koulov et al. 2003), we demonstrated that a family of small molecules derived from cholic acid termed 'cholapods' bind anions with high-affinity, promote Cl- efflux from liposomes and ion transport across polarised MDCK epithelia (Koulov et al. 2003). Using excised inside-out membrane patches from giant liposomes, we compared the activity of cholapods with that of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel under the same conditions. Addition of cholapods caused a concentration-dependent increase in Cl- current, but no unitary events were observed.

We present here results obtained with a series of cholapods studied in planar lipid membranes (PLM). Membranes were formed by painting a mixture of POPE/Cholesterol (7/3) plus a known amount of cholapod. Using PLM, we have access to both sides of the membrane and thus to modify its environment. Thus, it has been possible to measure cholapod-mediated current under a series of conditions. Addition of cholapods in DMSO to the cis side of the membrane increased the observed current at an applied voltage (e.g. -100 mV). As the ion concentration was elevated the magnitude of cholapod-induced current saturated. Permeability experiments have been carried out and the transporters show good anion vs. cation selectivity, and also selectivity between anions. We interpret these data to suggest that cholapods can mediate anion transport across artificial lipid membranes by a carrier mechanism. Access to both sides of the membrane makes the PLM a potent technique to analyse the mechanism of action of synthetic anion transporters.

Koulov AV et al. (2003). Angew Chem Int Ed Engl 42, 4931-4933.

Supported by the BBSRC, EU, CF Trust.

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