C15

The basolateral role in urinary concentration of mUT-A3 in the mouse kidney

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Facilitative UT-A and UT-B urea transporters regulate the movement of urea across biological membranes and play a central role in the urinary concentrating mechanism (Smith & Rousselet, 2001). We have previously reported that at least three different UT-A isoforms (mUT-A1, mUT-A2 and mUT-A3) are present in the mouse kidney (Fenton et al. 2002a). We have also shown that mUT-A3 mRNA levels are increased during thirsting (Fenton et al. 2002b). The aim of this study was to identify the specific renal location and function of mUT-A3.

MQ2, a novel antibody targeted to the C-terminal of mUT-A3, was raised in rabbits. Tissue for Western analysis was obtained from humanely killed male adult NMR1 mice. For immunolocalisation studies, mice were anaesthetized with Inactin (100 mg kg⁻¹ i.p.) before perfusion–fixation of kidneys with 4% paraformaldehyde. Western analysis of dissected mouse kidney protein showed that MQ2 only detected mUT-A3 (a 45–55 kDa protein). This isoform specificity is in contrast to our previously reported antibodies, ML446 and ML194 (Stewart et al., 2002). Immunolocalisation studies using MQ2 showed that mUT-A3 was only present in inner medullary collecting duct (IMCD) segments IMCD2 and IMCD3, and was located on the basolateral membranes. Finally, using 35Cl-labelled urea, Xenopus oocyte flux experiments confirmed that mUT-A3 was stimulated by short-term exposure to PKA stimulants (P < 0.01, ANOVA, n = 6).

These results indicate renal mUT-A3 is a basolateral transporter, regulated in the short-term by PKA. We therefore suggest that mUT-A3 plays an important role in transcellular urea transport across IMCD epithelia. Our findings also agree with reports that mUT-A3 plays an important role in transcellular urea transport regulated in the short-term by PKA. We therefore suggest that mUT-A3 is a basolateral transporter, (Morgan et al. 2002a,b). Other proteins (e.g. polycystin-1 and polycystin-2), mutation of which also causes renal cystic disease, are also expressed in the primary cilium and are involved in Ca²⁺ signalling (Nauli et al. 2003). To establish whether Ca²⁺ signalling is disrupted in inv cells, we plan to use apically located Ca²⁺-activated Cl⁻ channels (CACC) as sensitive detectors of intracellular Ca²⁺ concentration. As a first step, it is necessary to exclude the possibility that expression and/or regulation of CACC per se is altered by the inv genotype.

Renal collecting ducts were isolated from wild-type (WT), heterozygote (Het) and homozygous (Hom) inv mice (which were humanely killed) and cultured in OPTI-MEM 1 medium. Primary epithelial cells growing out from the ducts were used for patch clamping after 2–4 days. Capacitance measurements were (means ± s.e.m.): WT, 17.0 ± 4.2 pF (n = 3 cells); Het, 14.1 ± 0.7 pF (n = 4 cells); Hom, 12.4 ± 1.3 pF (n = 7 cells), indicating no genotypic related differences in cell surface area. Baseline currents were normally small (< 15 pA pF⁻¹ at ± 60 mV) in all three genotypes. Ionomycin (1 μM) evoked large Cl⁻-selective currents with time-dependent kinetics typical of CACC (WT, 166.5 ± 40.2 pA pF⁻¹ and −111.2 ± 26.9 pA pF⁻¹; Het, 274.6 ± 87.5 pA pF⁻¹ and −186.3 ± 54.3 pA pF⁻¹; Hom, 161.2 ± 26.2 pA pF⁻¹ and −130.3 ± 21.6 pA pF⁻¹ at ± 60 mV respectively with n values as above). There were no significant differences in CACC density between the genotype groups (P > 0.05, ANOVA). In four paired experiments using homozygous inv cells, ionomycin increased currents to 112.9 ± 21.5 pA pF⁻¹ and −93.0 ± 18.7 pA pF⁻¹ at ± 60 mV respectively. Subsequent reduction of bath Ca²⁺ from 2 mM to 0.1 μM (maintaining ionomycin at 1 μM) caused current density to decline to 6.6 ± 2.8 pA pF⁻¹ and −7.9 ± 2.4 pA pF⁻¹ at ± 60 mV, respectively.

We conclude that homozygous inv cells have a normal complement of CACC and that the channels can be activated normally by the intracellular Ca²⁺ signals generated by 1 μM ionomycin.


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All procedures accord with current UK legislation.

C16

Ca²⁺-activated Cl⁻ currents in renal collecting duct epithelia from inv mice


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The inv mouse exhibits reversal of the left–right body plan and develops renal cysts (Yokoyama et al. 1993). The protein encoded by the invs gene, inversin (Morgan et al. 1998), binds calmodulin and is highly expressed in the primary ciliation of renal cells (Morgan et al. 2002a,b). Other proteins (e.g. polycystin-1 and polycystin-2), mutation of which also causes renal cystic disease, are also expressed in the primary cilium and are involved in Ca²⁺ signalling (Nauli et al. 2003). To establish whether Ca²⁺ signalling is disrupted in inv cells, we plan to use apically located Ca²⁺-activated Cl⁻ channels (CACC) as sensitive detectors of intracellular Ca²⁺ concentration. As a first step, it is necessary to exclude the possibility that expression and/or regulation of CACC per se is altered by the inv genotype.

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We conclude that homozygous inv cells have a normal complement of CACC and that the channels can be activated normally by the intracellular Ca²⁺ signals generated by 1 μM ionomycin.


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PC24

Expression of Na⁺-dependent bicarbonate cotransporter (NBC3) and anion exchanger isoforms in mouse inner medullary collecting duct cells (mIMCD-K2)

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Our previous studies using mouse renal inner medullary collecting duct cells (mIMCD-K2 cell-line) have shown the presence of sodium–potassium–2 chloride (NKCC1) cotransport at the basolateral membranes (Glanville et al. 2001). However forskolin-stimulated secretory short-circuit current in a bicarbonate-containing medium was largely insensitive to 0.2 mM furosemide suggesting that alternative mechanisms must exist at this membrane to accumulate cytosolic Cl⁻ above its
electrochemical equilibrium value (Glanville et al. 2001). Accordingly we have sought evidence for expression and activity of alternative transporters such as the Na⁺-dependent bicarbonate cotransporter (NBC) and anion exchanger (AE) isoforms.

Isoform-specific polymerase chain reaction (PCR) primers were designed for mouse NBC1-3 and AE1-3 isoforms. Reverse-transcription/PCR and agarose gel electrophoresis of products showed products of the expected size for only NBC3 and AE2/3. The molecular identity of each product was confirmed by T/A cloning (Invitrogen) and sequencing of each product.

Using BCECF-loaded mMCDK2 cell monolayers perfused separately across apical and basolateral surfaces (Glanville et al. 2001), a change in the apical superfusion from a Na⁺-rich bicarbonate-free Krebs solution to one containing 10 mM bicarbonate resulted in cell acidification by 0.35 ± 0.06 pH units (mean ± s.e.m., n = 4), in contrast to the basolateral surface cell alkalisation (0.13 ± 0.02 pH units, n = 4) was observed. In Na⁺-free conditions, apical superfusion with 10 mM bicarbonate Krebs still resulted in cell acidification, but at the basolateral surface superfusion with 10 mM bicarbonate Krebs now resulted in cell acidification (0.26 ± 0.01 pH units). These data are consistent with basolateral expression of NBC-3. Upon superfusion of the basolateral surface with a low Cl⁻ (20 mM, Cl⁻ replaced by gluconate) bicarbonate-containing (10 mM) Krebs solution a cell alkalisation of 0.14 ± 0.02 pH units (n = 5) was observed whereas at the apical surface a small acidification was observed (0.06 ± 0.01 pH units (n = 5)). These data are consistent with AE expression only at the basolateral surface.

In conclusion we propose that the co-expression of NBC-3 isoform-specific polymerase chain reaction (PCR) primers were designed against the original partial sequence. The full-length clone (1.8 kb) was sequenced (Lark Technologies) and is predicted to encode a 370 amino acid protein (zROMK2), 54% identical to rat ROMK2. The full-length cDNA was sub-cloned into pcDNA3 and used to transcribe cRNA (mMessage mMachine™ -Ambion) which was expressed in oocytes isolated by standard methods from humanely killed Xenopus laevis (n = 3). Stage V and VI oocytes were injected with 50 nl of H₂O (control) or 50 nl H₂O containing 50 ng (1 ng nl⁻¹) of cRNA encoding zROMK2 or 1 ng (0.02 ng nl⁻¹) of cRNA encoding rat ROMK2. The oocytes were incubated at 18°C, and functional expression was assessed by two microelectrode voltage clamp 3–4 days following injection.

At an external [K⁺] of 2.00 mM, oocytes injected with rat ROMK2 displayed a resting membrane potential (Vᵢₐₕₐₜ) of -96.00 ± 1.36 mV (mean ± s.e.m., n = 16), and at a clamp potential of 0 mV an outward current (Iₒ) of 3.15 ± 0.16 μA which was sensitive to 5 mM Ba⁺⁺ (1.47 ± 0.07 μA; P < 0.001, Student’s paired t test). Similarly, in oocytes expressing zROMK2 Vᵢₐₕₐₜ was 92 ± 1.52 mV and Iₒ was 1.93 ± 0.72 μA (n = 16) which was also sensitive to Ba⁺⁺ (0.72 ± 0.09 μA; P < 0.001). Control oocytes displayed a Vᵢₐₕₐₜ of -26.20 ± 0.76 mV and Iₒ of 0.13 ± 0.76 μA (n = 12) which was unaffected by Ba⁺⁺ (0.09 ± 0.01 μA).

These results suggest that adult Danio rerio express a ROMK2-like potassium selective channel. A consideration of the predicted zROMK2 amino acid sequence indicates that there are likely to be differences in the regulatory mechanisms controlling the activity of this channel. This, along with the sites of expression and physiological role of this protein require further study.


We thank the National Kidney Research Fund for financial support. S.H. is a graduate student supported by the Iranian ministry of Health and Medical education and Arak medical university.

All procedures accord with current UK legislation.

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**PC25**

**Functional expression of zebrafish (Danio rerio) ROMK2 (Kir1.1b)**

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The zebrafish is a model organism of increasing importance in the study of human congenital and inherited disease (Briggs, 2002). However, little information regarding cellular and molecular function in this organism is available. The nephron of the teleost kidney lacks a loop of Henle, but does posses a distal tubule which appears to act in an analogous fashion to the mammalian diluting segment (Nishimura et al. 1983). Here we report the successful expression of a ROMK-related channel from this species.

A search of the NCBI GENBANK database identified a partial sequence of high similarity to rat ROMK2 for a clone from an adult Danio rerio cDNA library (Image Consortium ID: 3815817). To verify the identity of this clone, a PCR reaction was performed using primers which were designed against the original partial sequence. The full-length clone (1.8 kb) was sequenced (Lark Technologies) and is predicted to encode a 370 amino acid protein (zROMK2), 54% identical to rat ROMK2. The full-length cDNA was sub-cloned into pcDNA3 and used to transcribe cRNA (mMessage mMachine™ -Ambion) which was expressed in oocytes isolated by standard methods from humanely killed Xenopus laevis (n = 3). Stage V and VI oocytes were injected with 50 nl of H₂O (control) or 50 nl H₂O containing 50 ng (1 ng nl⁻¹) of cRNA encoding zROMK2 or 1 ng (0.02 ng nl⁻¹) of cRNA encoding rat ROMK2. The oocytes were incubated at 18°C, and functional expression was assessed by two microelectrode voltage clamp 3–4 days following injection.

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These results suggest that adult Danio rerio express a ROMK2-like potassium selective channel. A consideration of the predicted zROMK2 amino acid sequence indicates that there are likely to be differences in the regulatory mechanisms controlling the activity of this channel. This, along with the sites of expression and physiological role of this protein require further study.


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**PC26**

**Prostaglandins E₂ and I₁ increase basal water permeability in isolated rat inner medullary collecting ducts**

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Aquaporin-2 (AQP2) water channels are shuttled from intracellular vesicles to the apical plasma membrane of renal collecting duct principal cells in response to vasopressin (AVP). This shuttling response can be modulated by a variety of local and systemic factors, including prostaglandins. Previous reports have suggested that prostaglandins may increase collecting duct water permeability in their own right, but inhibit the response to AVP, and there has been considerable debate about the receptors involved (Veis et al. 1990; Hebert et al. 1993). In the present study, we have investigated the ability of PGE₂ and PGI₁ to cause shuttling of AQP2, alone and in conjunction with AVP. For each experiment, two male Wistar rats (200–250 g) were anaesthetised with pentobarbitone sodium (240 mg kg⁻¹ i.p.) and killed by cervical dislocation. The kidneys were rapidly removed and inner medullary tubule suspensions prepared as previously described (Shaw & Marples, 2002). The pooled suspension was divided into six aliquots, and incubated at 37°C for 20 min with: control, AVP (1 nm), PGE₂ (280 nm), PGI₁ (280 nm), PGE₂ + AVP, and PGI₁ + AVP. The tissue was then homogenised, and plasma membrane- (PM) and intracellular
vesicle- (ICV) enriched fractions prepared for Western blotting with an antibody against the c-terminus of AQP2. After visualisation by enzyme chemiluminescence (ECL), the PM:ICV ratio of the control for each experiment was set to 100%, and other values expressed relative to this. Thus an increase in the PM:ICV ratio demonstrates an increase in the fraction of AQP2 present in the plasma membrane. Data, expressed as a percentage of control, are shown as means ± S.E.M., and were analysed using Student’s unpaired t test, and considered significant if $P < 0.05$.

In all experiments, AVP caused a shift in the PM:ICV ratio ($189 ± 13\%$ of control, $n = 7$, $P < 0.001$). Both PGE$_2$ ($133 ± 11\%$, $P < 0.05$) and PGI$_2$ ($160 ± 20\%$, $P < 0.05$) induced a modest but significant shift on their own. In neither case did the addition of AVP cause a significant further shift (PGE$_2$ +AVP $161 ± 19\%$ n.s., PGI$_2$ +AVP $260 ± 45\%$ n.s.), although it is also the case that the increase in ratio caused by AVP is not significantly diminished by PGI$_2$.

These results indicate that either PGE$_2$ and PGI$_2$ alone can induce shuttling of AQP2 to the apical plasma membrane, while PGE$_2$, but probably not PGI$_2$, impairs the ability of AVP to induce such shuttling. It remains to be determined which receptor types mediate the various effects of these prostaglandins.


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*All procedures accord with current UK legislation.*