C1

Calcium-sensing receptor activation induces protein kinase C-mediated feedback phosphorylation at residue Thr-888

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Extracellular Ca^{2+} homeostasis is maintained by the suppressive action of the calcium-sensing receptor (CaR) on parathyroid hormone secretion and renal Ca^{2+} reabsorption. Substitution of the CaR putative protein kinase C (PKC) consensus sequence, Thr-888, alters significantly the function of the receptor expressed in HEK-293 cells, whilst the CaR-induced suppression of PTH secretion is modulated by treatment with phorbol esters and PKC inhibitors. Therefore, to investigate CaR phosphorylation directly we developed an affinity-purified, phospho-specific polyclonal antibody that recognises the phosphorylated form of CaR_{T888}. Using this antibody, we examined PKC-mediated CaR_{T888} phosphorylation in human CaR-transfected HEK cells by immunoblotting and immunofluorescence.

Acute treatment of the cells with the phorbol ester PMA increased the immunoreactivity of two bands corresponding to the immature (140kDa) and mature (160kDa) glycosylated CaR and increased total cellular immunoreactivity by immunofluorescence. These signals could be blocked selectively using the phosphorylated but not the non-phosphorylated immunising peptide and were absent in cells expressing a mutant CaR lacking Thr-888 (CaR_{T888A}).

Increasing the concentration of extracellular Ca^{2+} (Ca^{2+}_o) from 0.5 to 2.5mM induced a small rise (+26±18%, ±sem) in CaR_{T888} phosphorylation. Increasing it to 5mM, or, addition of the calcimimetic (i.e. CaR positive allosteric modulator) NPS-467R (1μM; +42±10%, $P<0.05$ ANOVA, N=4) further elevated the response, the effect of NPS-467R being stereoselective. The CaR agonist-induced increase in CaR_{T888} phosphorylation was localised to the cell membrane as demonstrated by confocal microscopy and was sensitive to PKC inhibition using GF109203X (-60±3%, $P<0.001$). No responses were seen in empty vector-transfected HEK cells. Furthermore, in Fura2-loaded CaR-HEK cells, phorbol ester treatment abolished 2.5mM Ca^{2+}_o-induced Ca^{2+}_i oscillations while GF109203X, or chronic PMA pre-treatment, converted the oscillatory response to a sustained plateau response. In addition, the agonist-sensitivity of CaR_{T888A} was enhanced relative to wild-type CaR. Finally, following the removal of an acute PMA pretreatment, the CaR_{T888} residue was dephosphorylated 61% (±13%, $P<0.05$) within 5 min, a process that was completely blocked by the protein phosphatase 1/2A inhibitor calyculin (100nM).

Together these data prove that the CaR can be phosphorylated by PKC on residue Thr-888 and that agonist stimulation of the receptor elicits feedback phosphorylation of this residue. By this mechanism it may be possible to alter the physiological sensitivity of the CaR and thus affect calcium homeostasis.

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

C2

AQP2 shuttling in $mpkCCD_{c14}$ cells

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The $mpkCCD_{c14}$ cell line is derived from mouse cortical collecting duct, and expresses aquaporin 2 (AQP2) endogenously, in a vasopressin (VP)-regulated fashion (Hasler *et al.* 2002). In the present study we have investigated whether these cells shuttle AQP2 to the plasma membrane in response to VP. We have also investigated the effect of angiotensin II (AII), which we have previously shown to cause shuttling in collecting ducts.

Cells were grown on Falcon cell culture inserts in modified DMEM:F-12 medium (Hasler *et al.* 2002), until transepithelial resistance was maximal. They were then kept for 24 h in serum- and hormone-free medium, followed by 24 h in the same medium supplemented with 1 nM VP. After a 1 h wash to cause internalisation of AQP2, cells were treated with nothing, VP, or AII (100 pM) for 30 min. For confocal microscopy, cells were then fixed, incubated with fluorescein-tagged wheat germ agglutinin to mark the apical surface, permeabilized, and labelled with an antibody against the c-terminus of AQP2, with a Cy3-tagged secondary antibody. For semi-quantitative measurements of AQP2 localisation, cells were homogenised for membrane fractionation and used for Western blotting. For measurement of cAMP levels, cells were lysed in 0.1 M HCl for use with the Sigma cAMP ELISA. Results were analysed using Student's t tests and the false-discovery rate procedure, and data expressed as mean ± S.E.M.

Western blotting demonstrated that AQP2 shuttles from intracellular vesicles to the plasma membrane in response to vasopressin (268 ± 44% vs. control, n=4, $p<0.05$). Confocal microscopy demonstrated that AQP2 was predominantly intracellular in control cells, and predominantly apical after VP. VP also caused an increase in cAMP levels (35 ± 5 pmol/ml vs. 11 ± 1 pmol/ml, n=6, $p<0.05$). Results indicate that treatment with AII did not cause a significant increase in the shuttling of AQP2, although it did increase slightly compared to controls. Confocal microscopy also showed some areas of apical AQP2 labelling with AII treatment, although some areas remained intracellular. However, AII did not cause an increase in cAMP levels.

These results demonstrate that this cell line is a valuable tool for investigating the acute regulation of AQP2 distribution, as well as the long-term changes in expression previously described, and that AII may induce AQP2 shuttling to a very slight extent, although apparently not via cAMP formation.

Hasler U *et al.* (2002). *J Biol Chem* **277**, 10379-10386.

Terris J *et al.* (1995). *Am J Physiol Renal Physiol* **269**, F775-F785.

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

C3

Immunofluorescent labelling reveals dDAVP-dependent P2 receptor expression and apical P2 receptor-mediated inhibition of AQP2 expression in mpkCCD(cl4) cultures

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In the renal collecting duct (CD), water reabsorption is determined by the expression of aquaporin-2 (AQP2) in the apical membrane of principal cells, which in turn is dependent on the activation of vasopressin receptors in the basaolateral membrane. This process is inhibited by extracellular ATP, which acts on P2 receptors expressed on the basolateral membrane of CD principal cells, and there is evidence that $P2Y_2$-like receptors are responsible (Unwin *et al.* 2003). The present study has used immortalized mouse mpkCCD(cl4) CD principal cells to investigate whether other P2 receptor subtypes may influence vasopressin-induced AQP2 expression.

Cultured mpkCCD(cl4) cells were grown to confluence on transwell permeable supports, then treated with 1 nM dDAVP (applied to the basal media) for 96 h to induce AQP2 expression. In some experiments, cells were also treated with 1 μM ATPγS (applied to the apical media) for the final 2 h prior to termination of dDAVP treatment. Confluent monolayers were then washed in phosphate-buffered saline and fixed with paraformaldehyde (4% for 10 min) before performing immunofluorescence labelling (using P2- and AQP2-specific antibodies) and confocal microscopy to investigate AQP2 and P2 receptor expression patterns.

In monolayers of cells untreated with dDAVP (n=5), immunostaining for AQP2 was not evident. In contrast, positive staining for $P2Y_1$ and $P2Y_4$ receptors was seen in the apical region, whereas $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptor subunits and $P2Y_2$, $P2Y_{11}$ and $P2Y_{12}$ receptors were located throughout the cytoplasm up to the cell borders and more strongly in perinuclear regions. Treatment with dDAVP (n=5) resulted in positive immunostaining for AQP2, $P2X_1$ and $P2X_2$ in the apical region and basolateral staining for $P2Y_2$. Staining for all other P2 receptors remained unchanged. In monolayers treated with dDAVP and ATPγS (n=3), AQP2 and $P2X_1$ staining in the apical region was weak and vesicular, whereas all other P2 receptor staining remained unchanged.

These results suggest that dDAVP may regulate the expression of some P2 receptors ($P2X_1$ apically, $P2X_2$ apically and $P2Y_2$ basolaterally), as well as AQP2. They also suggest that apically located P2 receptors ($P2Y_1$, $P2Y_4$, $P2X_1$ and/or $P2X_2$) might play a role in modulating vasopressin-stimulated water reabsorption in the CD by inhibiting the expression or trafficking of AQP2. These findings thus reinforce the suggestion that intraluminal nucleotides might act as paracrine/autocrine agents in the nephron.

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

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

C4

Combined loop diuretic and mineralocorticoid treatment as a screening test for distal renal tubular acidosis

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The standard test for the diagnosis of distal renal tubular acidosis (dRTA) is to determine whether urine pH falls appropriately (to <5.5) in response to the administration of an acid load in the form of oral NH_4Cl, usually given as a single dose (Wrong & Davies, 1959). However, NH_4Cl capsules in the quantities required are unpleasant to ingest and frequently lead to vomiting and abandonment of the test. The present study has assessed an alternative and more palatable test of urinary acidification.

The loop diuretic frusemide increases the delivery of sodium to the distal tubule and collecting ducts, which stimulates electrogenic sodium reabsorption in those nephron segments, thereby favouring H^+ secretion (Batlle, 1986). In our study, frusemide was combined with the synthetic mineralocorticoid fludrocortisone, which, in addition to stimulating sodium reabsorption, directly stimulates H^+ secretion by α-intercalated cells. Ten patients with previously diagnosed dRTA were given orally, on separate occasions and in random order, either NH_4Cl capsules (100 mg/kg) or fludrocortisone (1 mg) plus frusemide (40 mg). Urine was collected hourly for the next 8 h and its pH measured with a glass electrode. This protocol was repeated in a control group of subjects comprising five healthy volunteers and five patients with recurrent renal calculi.

Of the original cohort of subjects, one control and two dRTA patients were unable to complete the study due to vomiting after NH_4Cl loading; no one experienced side effects from the frusemide/fludrocortisone test. In the remaining members of the control group (n=9), urinary pH fell below 5.5 within 7 h in all subjects with the frusemide/fludrocortisone test (minimum pH = 4.79 ± 0.30; mean ± SEM) whereas 3 control subjects failed to acidify their urine below pH 5.5 with the NH_4Cl test (minimum pH = 5.23 ± 0.45). This difference was statistically significant ($P<0.05$, Student's paired t test). In the dRTA group (n=8), no patient was able to lower their urinary pH to 5.5 after either test (frusemide/fludrocortisone, minimum pH = 6.75 ± 0.28; NH_4Cl, minimum pH = 6.50 ± 0.40; NS).

We conclude that the combination of frusemide and fludrocortisone provides an effective, well-tolerated alternative to the standard NH_4Cl urinary acidification test.

Batlle DC (1986). Kidney Int 30, 546-554.

Wrong O & Davies HE (1959). Q J Med 28, 259-313.

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

PC9

Extracellular signal-related kinase (ERK) activity is required for growth of MDCK-derived cysts

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Growth of renal cysts results from both renal epithelial cell proliferation and fluid accumulation within the cyst lumen, and cAMP has a central role in this process. Inhibition of G protein coupled P2Y receptors upstream of cAMP can reduce growth of MDCK-derived cysts (1). Activation of P2Y receptors can increase intracellular [Ca^{2+}] and stimulate cAMP activity, both of which are important signalling events for the extracellular signal-related kinase (ERK) cascade. We have investigated the activity of ERK on growth of MDCK-derived cysts.

MDCK cells were cultured in collagen gel in the presence of the cAMP agonist forskolin to stimulate cyst formation. The ERK pathway was inhibited upstream using the MEK inhibitors PD98059 (50 μM) or U0126 (10 μM). Cyst diameter was measured directly from sequential photographs and cyst volume calculated. MDCK cysts were harvested from collagen gels by centrifugation (12 000 g, 5 min), washed 3 times in Dulbecco's PBS and the pellet resuspended in Ripa buffer. Fifty μg of protein was electrophoresed on 12% SDS-PAGE gels and activity of ERK was measured by immunoblot. Results are expressed as means ± SEM of n observations for a total of three experiments per treatment. One-way ANOVA was used to compare data sets and differences were considered statistically significant if $p<0.05$.

In control MDCK cysts, growth rate was 1.2 ± 0.16 nl/day (n=28 cysts). Inhibition of the ERK pathway resulted in a 90% reduction in cyst growth (PD98059 0.12 ± 0.02 nl/day, $P < 0.001$, n=36 and U0126 0.14± 0.02 nl/day, $P < 0.001$, n=41). Phospho-ERK activity increased over time on days 6 and 9, and was maximal on day 12 when compared with non-phosphorylated ERK.

These data strongly suggest the ERK signal transduction pathway for cell proliferation is a key component of growth of MDCK cysts.

Turner et al. (2003). Am Soc Nephrol abstract PO211.

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

PC10

Relaxin-induced changes in renal function of the female rat

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There is growing evidence which suggests that the ovarian hormone relaxin (RLX) contributes to the changes in renal function associated with pregnancy. Studies in male rats have shown that RLX induces an increase in renal blood flow and glomerular filtration rate (1) as well as a diuresis and natriuresis following long- (7 days) but not short-term (2h) administration (2). The aim of this study was to determine whether RLX has similar effects in female rats and to localise expression of the RLX receptor LGR7 in the kidney. Under isoflurane anaesthesia (4% in O_2, 2 l/min), female Sprague Dawley rats were implanted with an osmotic minipump containing human recombinant RLX (0.4 μg/h or 4 μg/h) or vehicle (20 mM sodium acetate). Seven days later, standard renal clearance measurements were made under Inactin anaesthesia (100 mg/kg thiobutabarbital sodium, i.p.). A servo-controlled fluid replacement system was used to maintain a euvolaemic body fluid status. RLX (4 μg h^{-1} 100g bwt^{-1}) or vehicle was administered acutely to a second group of anaesthetised rats for a period of 2h under similar euvolaemic conditions. Kidneys were harvested post mortem from separate groups of female rats previously exposed to RLX at 0.4 μg/h or 4 μg/h for 7 days and pregnant female rats (Day 11, corresponding to maximal plasma [RLX]) for Western blotting and immunohistochemistry for the LGR7 receptor. Administration of RLX at 0.4 μg/h for 7 days induced a significant reduction in osmolar excretion and urinary sodium and chloride excretion and clearance rates. Plasma sodium (vehicle 126.5 ± 0.3 vs RLX 105.1 ± 0.8 mmol/l) and chloride (vehicle 112.8 ± 4.5 vs RLX 104.4 ± 2.7 mmol/l) concentrations and plasma osmolality (vehicle 292.6 ± 2.1 vs RLX 236.9 ± 6.1 mosmol/kg) were also reduced. RLX at 4 μg/h for 7 days or acutely for 2 h had no effect on these variables. Statistical significance was assumed at the 5% level.

Western blotting revealed LGR7 expression in both the cortex and medulla of control, RLX-treated and pregnant female rat kidneys. LGR7 expression was increased in the kidneys of pregnant rats (control 103±16 vs 217±15 arbitrary units, $n = 5$, $P<0.01$) and rats treated with RLX at 0.4 μg/h (178±42, $n = 5$, $P<0.05$) but not 4 μg/h. Immunolocalisation showed that LGR7 was present on the proximal tubule and inner medullary collecting duct, in addition to blood vessels. These data show that RLX evokes dose-related changes in renal function in the female rat which, at the lower dose employed, resemble those seen in pregnancy. The lack of effect at the higher dose is consistent with the known desensitisation of RLX receptors following prolonged administration or exposure to high doses (3). Up-regulation of LGR7 expression in kidneys from pregnant rats lends further support to the suggestion that RLX contributes to the changes in renal function seen in pregnancy.

Table 1. Renal function in female Sprague Dawley rats after 7 days of vehicle or RLX treatment

	Vehicle ($n = 6$)	RLX, 0.4 μg/h ($n = 6$)
GFR (ml min^{-1} 100g bwt^{-1})	0.60±0.09	0.64±0.10
UV (μl min^{-1} 100g bwt^{-1})	21.6±2.6	22.4±2.6
$U_{Na}V$ (μmol min^{-1} 100g bwt^{-1})	4.9±0.5	2.8±0.4**
$U_{Cl}V$ (μmol min^{-1} 100g bwt^{-1})	4.8±0.3	3.5±0.4*

Data are means ± SEM. Statistical comparisons were by Student's t test, *$P<0.05$; **$P<0.01$.

Danielson LA *et al.* (1999). *J Clin Invest* **103**, 525-533.

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Cheah SH & Sherwood OD (1981). *Endocrinology* **109**, 2076-2083.

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

PC11

Increased glomerular mRNA expression of the $P2X_7$ receptor in experimental glomerulonephritis is associated with an increase in p53 and bax

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The $P2X_7$ receptor, a member of the P2X receptor family of ATP-gated ion channels, is unique, since in addition to ion channel formation, it can also regulate the release of IL-1β via regulation of caspase-1 and induce membrane blebbing, leading to apoptosis. We have previously demonstrated increased expression of $P2X_7$ receptor mRNA on day 4 after nephrotoxic serum injection in glomeruli of rodent models of glomerulonephritis (GN) (1). This coincides with the onset of proteinuria and macrophage infiltration which are maximal on day 4 in this model.

Male Wistar Kyoto (WKY) rats weighing 200-250 g were injected with 2.5–10 mg of rabbit anti-rat glomerular basement membrane (GBM) globulin. At 2, 4 and 7 days after the nephrotoxic serum injection (n=6 for each time point) glomeruli were isolated from the renal cortex using a differential sieving technique and RNA was extracted for real-time PCR with gene-specific primers for the $P2X_7$ receptor p53, bax and bcl-2. Mouse renal tissue obtained previously (2) was used for the immunohistochemical localisation of P2X7 receptor protein and TUNEL staining as a marker of apoptosis. To compare sets of data, one-way ANOVA, followed by the Tukey-Kramer multiple comparisons post-hoc test, was used (Graphpad Instat version 3.06). Differences were considered statistically significant at $P<0.05$.

Expression levels were calculated as a ratio to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) and control values were normalized to 1.0. $P2X_7$ receptor mRNA expression was unchanged on day 2 after nephrotoxic serum injection (control 1.0 ± 0.16, day 2 0.85 ± 0.20), but increased maximally on day 4 (2.66 ± 0.44 P <0.01); on day 7 there was no significant increase (1.41 ± 0.21). Expression of the pro-apoptotic p53 and bax genes was increased on day 2 (p53 control 1.0 ± 0.22, day 2 2.50 ± 0.55 n.s. and bax control 1.0 ± 0.52, day 2 6.35 ± 1.58 P = 0.01) and maximally increased on day 4 (p53 4 4.81 ± 1.12 P <0.01 and bax 8.81 ± 2.24 P <0.01). By day 7, p53 and bax expression was still increased, but to a lesser degree (p53 2.6 ± 0.49, P = 0.01, and bax 2.06 ± 0.60 n.s.). Expression of the anti-apoptotic gene bcl-2 was unchanged at each time point. Results are expressed as mean ± SEM.

In summary, the pro-apoptotic $P2X_7$ receptor, as well as the p53 and bax genes, is maximally increased on day 4. These results suggest that glomerular expression of the $P2X_7$ receptor could be important in the pathogenesis of GN, perhaps through cell loss by apoptosis.

Tam et al. (2004). Renal Assoc abstract RA4544.

Tarzi RM, Davies KA, Claassens JW et al. (2003). Am J Pathol 162, 1677-1683.

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

PC12

An inwardly rectifying K^+ current in cultured mouse collecting duct cells: dependence on ATP hydrolysis

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K^+ channels expressed in the renal collecting duct have a number of roles, including K^+ secretion, maintaining the membrane potential and cell volume regulation (Giebisch & Wang, 1996). Previous studies have shown functional and molecular evidence for a number of different inwardly rectifying K^+ channels (Kir), which could be involved in these roles. The aim of the following study was to investigate the presence of Kir channels in a cultured mouse collecting duct cell line (M8).

K^+ currents were examined in whole cell patches with either 135 mM KCl or 130 mM NaCl solutions in the bath. The pipette contained a 145 mM KCl solution, which contained EGTA and no added Ca^{2+}. Clamp potential was stepped from a holding value of –40 mV, to between +100 and -100 mV in -20mV steps. In unpaired cells, the current sensitive to 5 mM barium (Ba^{2+}) was determined. Whole cell currents were recorded either in the control circumstance or in the presence of 100 μM ATP or a non-hydrolysable ATP derivative, adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP). On achieving the whole cell configuration currents increased to a peak, followed by a fall in magnitude to steady-state. The Ba^{2+}-sensitive current (I_{Ba}) was measured at the peak current level and again after 5 minutes. All values are expressed as means ± SEM. Statistical significance was tested using Student's paired and unpaired t tests and ANOVAs, and assumed at the 5% level.

In paired cells with symmetrical KCl solutions the Ba^{2+} - sensitive conductance demonstrated inward rectification, outward and inward conductances were 94.01 ± 27.28 μS/cm^2 and 158.91 ± 35.47 μS/cm^2, respectively (n = 11). With high NaCl in the bathing solution peak I_{Ba} at +100 mV was 24.2 ± 3.25 pA/pF. This fell to 12.6 ± 2.23 pA/pF (n = 32) over 5 min. In unpaired cells initial I_{Ba} with ATP and AMP-PNP in the pipette was 22.7 ± 5.11 pA/pF (n = 13) and 20.6 ± 2.66 pA/pF (n = 14) respectively. There was no significant difference between the peak current levels. Rundown of I_{Ba} was prevented with ATP in the pipette, but not by AMP-PNP. The shift in I_{Ba} with ATP, 3.09 ± 2.65 pA/pF (n = 13), was significantly different to the decrease observed in the absence of pipette ATP and in the presence of AMP-PNP, 11.6 ± 1.80 pA/pF (n = 32) and 9.69 ± 2.09 pA/pF (n = 14), respectively. AMP-PNP was not significantly different to the control circumstance.

These data indicate that cultured mouse collecting duct cells contain an inwardly rectifying K^+ conductance. The K^+ conductance is maintained by intracellular ATP, but not by AMP-PNP, suggesting that it is regulated via a mechanism dependent on ATP hydrolysis.

Geibisch G & Wang WH (1996). Kidney Int 49, 1624-1631.

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

PC13

Effect of urotensin II infusion on renal function in the anaesthetised rat

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Urotensin II (UII) is an 11-amino acid vasoactive peptide which has been identified as the ligand for the human orphan G protein-coupled receptor GPR-14, now designated the UT receptor. UII has been shown to stimulate epithelial sodium transport in fish [1] and there is now evidence that it also affects renal function in mammals. Recently, we have shown that bolus injections of rat UII (rUII) into the anaesthetised rat produced dose-related reductions in glomerular filtration rate (GFR), urine flow rate and sodium excretion [2]. The aim of the current study was to determine the effect of a continuous infusion of rUII on renal function in the rat. Anaesthetised (Intraval, 100 mg/kg, i.p.) male Sprague Dawley rats (n=7 per group) were infused with 0.154M saline (containing ^{3}H inulin as a marker of GFR and *para*-aminohippuric acid as a marker of effective renal plasma flow, ERBF) via a jugular vein catheter for 4 hours at 50 µl/min. Animals were then divided into three groups which received either vehicle, rUII at 0.6 or 6 pmol min^{-1} (100g body weight)$^{-1}$ for 1 hour. The infusate was then switched back to saline for a further hour. Urine samples were taken every 15 min via a bladder catheter; blood samples were taken once per hour via a carotid artery catheter for the measurement of electrolyte concentrations. All measured variables were in a steady state and did not differ between groups prior to the infusion of rUII; mean arterial pressure was unaltered throughout. Compared with the vehicle-infused group, rUII at 0.6 and 6 pmol min^{-1} (100g body weight)$^{-1}$ caused significant (P<0.05) dose-related decreases in glomerular filtration rate (GFR), which reached a maximum 45 min after the start of infusion (Table 1). rUII at 6 pmol min^{-1} (100g body weight)$^{-1}$ also induced significant (P<0.05) reductions in effective renal blood flow (ERBF), urine flow (UV) and urinary sodium excretion rate ($U_{Na}V$). Fractional excretion of sodium (FE_{Na}) was increased (P<0.01) during infusion of rUII at 0.6 but not 6 pmol min^{-1} (100g body weight)$^{-1}$.

Urinary K^+, Ca^{2+} and Mg^{2+} clearance and excretion rates also fell during rUII infusion at 6 pmol min^{-1} (100g body weight)$^{-1}$. These data suggest that rUII, at the doses employed in this study, influenced renal function by reducing GFR and hence the filtered load of ions. The pronounced haemodynamic effect of rUII may mask an additional tubular action, as FE_{Na} was increased at the lower dose of rUII employed. This is consistent with our previous observation that the UT receptor is expressed in the loop of Henle and inner medullary collecting ducts [2].

Table 1. Renal variables after 45 mins i.v. infusion of rUII at either 0.6 or 6 pmol min^{-1} (100g body weight)$^{-1}$

	Vehicle	rUII (0.6 pmol min^{-1} 100g^{-1})	rUII (0.6 pmol min^{-1} 100g^{-1})
GFR (ml min^{-1} 100g^{-1})	1.0 ± 0.2	0.7 ± 0.2*	0.3 ± 0.1*
ERBF (ml min^{-1} 100g^{-1})	3.2 ± 0.5	2.9 ± 1.3	0.9 ± 0.4*
UV (µl min^{-1} 100g^{-1})	23.8 ± 4.8	26.9 ± 12.4	9.8 ± 5.2*
$U_{Na}V$ (µmol min^{-1} 100g^{-1})	2.7 ± 0.7	3.2 ± 1.6	1.1 ± 0.6*
FE_{Na} (%)	2.2 ± 0.2	3.7 ± 0.6**	2.8 ± 0.5

Data presented as mean ± S.E.M. *P<0.05, **P<0.01 vs. vehicle group, one-way ANOVA.

Balment RJ *et al.* (2005). *Ann NY Acad Sci* **1040**, 66-73.

Song W *et al.* (2006). *Kidney Int* **69**, 1360-1368.

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

PC14

Membrane targeting of ROMK Bartter's mutants in polarized MDCK cells

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Bartter's syndrome comprises a group of renal tubular disorders characterised by salt and fluid wasting and associated hypotension (Hebert *et al.* 2005). Mutations in the gene *KCNJ1*, which encodes the small conductance K^+ channel ROMK (Kir 1.1), are responsible for Type II Bartter's syndrome. ROMK mediates K^+ recycling across the apical membranes of the thick ascending limb (TAL) and K^+ secretion in the distal nephron. Characterisation of mutations which disrupt channel function and/or trafficking and targeting contribute much to the understanding of ROMK physiology. A previous study (Peters *et al.* 2003) indicated that the trafficking of four Bartter's mutants (T71M, V72E, A306T and F325C) was disrupted in non-polarized HEK 293 cells. In the present study, we investigated the properties of these mutants in polarized MDCK II cells in order to determine whether they also disrupt targeting.

The cDNAs encoding human ROMK1 and Bartter's mutants had been previously N-terminally tagged with eGFP(Peters *et al.* 2003). Confluent, polarized MDCK II cells grown on permeable filter supports were transiently transfected using Lipofectamine 2000^{TM} with 1µg cDNA encoding wild-type ROMK1 or ROMK1 Bartter's mutants. At 48h post transfection, the monolayers were fixed with methanol:acetone (7:3 v/v) and nuclei were counterstained with 5 µg/ml prodium iodide (red). Intracellular localisation of the eGFP-fusion proteins was determined by fluorescence confocal microscopy. Transfections were repeated between 3 and 5 times for each construct.

As expected, WT-ROMK1 was predominantly targeted to the apical pole in polarized MDCK II cells. The T71M (N-terminus) and A306T (C-terminus) mutants targeted to the apical domain and the fluorescence distribution pattern was similar to WT. In contrast, the V72E and F325C mutants showed a fluorescence pattern consistent with intracellular localisation and there was no distinct expression at either membrane domain.

These results indicate that single amino acid mutations in either the N- and C-termini can have profound effects on trafficking and targeting, and underscore the importance of both termini in the ability of ROMK to be targeted normally.

Hebert SC *et al.* (2005). *Physiol Rev* 85, 319-371.

Peters M *et al.* (2003). *Kidney Int* 64, 923-932.

We thank Kidney Research UK for financial support. V.M.C. was a recipient of a White Rose scholarship. The cDNA encoding the Bartter's mutants were kindly provided by Dr M. Peters (Marburg).

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

PC15

A high-salt diet increases the expression of NADPH oxidase in the Wistar-Kyoto rat kidney

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Oxidative stress is determined by the balance between the generation of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and the antioxidant defence systems such as superoxide dismutase (SOD). O_2^- is produced in activated phagocytes by the enzyme NADPH oxidase. NAD(P)H oxidase has also been implicated in O_2^- production in the vasculature and in the kidney cortex and medulla. A high salt intake enhances oxidative stress in rat skeletal muscle arterioles and vessels and increases blood pressure, protein excretion and renal fibrosis and worsens renal function in several models of chronic renal failure and accelerates the decline of renal function in patients with chronic renal failure (1). It was recently shown that a high salt diet induces oxidative stress in the rat kidney. Furthermore quantitative-RT-PCR experiments showed an increased expression of mRNA for the NAD(P)H oxidase components gp91phox and p47phox and decreased expression of mRNA for the Cu/Zn and Mn isoforms of SOD in the renal cortex (2).

To expand on these findings, our aim in this study was to determine the effects of a high salt diet on protein expression levels of the gp91phox subunit of NADPH oxidase and the Cu/Zn and Mn isoforms of SOD in both the renal cortex and medulla using Western blotting. Three month old male Wistar-Kyoto rats were assigned to two groups (n = 3) and were placed on either normal (0.03% Na^+) or high salt (0.9% Na^+) rat chow diet for 14 days. The animals were then killed with an overdose of anaesthetic (chloralose/urethane) and kidneys were immediately removed. Following dissection of cortex and medulla, tissue samples were homogenised in radio-immunoprecipitation (RIPA) buffer and following centrifugation, protein concentrations of supernatants were determined by Bradford assay. Protein samples (20 μg) were resolved by 12% SDS-polyacrylamide gel electrophoresis, electro-transferred to nitrocellulose membranes and probed with polyclonal antibodies specific for Cu/Zn SOD, Mn SOD, gp91phox and actin (loading control). Following detection of antibody binding by chemiluminescence, band intensities were quantified by densitometry. To correct for variations in protein loading, Cu/Zn SOD, Mn SOD, gp91phox band intensities were divided by corresponding actin band intensities. Means±SEMs were calculated for the high salt and normal salt groups and comparisons were undertaken using unpaired Student's t test. Significance was taken when $P < 0.05$.

Our result show that cortical tissue from normal and high salt fed rats contained levels of gp91phox at a relative band intensities of 0.31 ± 0.05 and 0.67 ± 0.04, respectively, a 2.2-fold increase (n=3, $P < 0.05$). Similarly, medullary tissue from normal and high salt fed rats contained levels of gp91phox at a relative band intensities of 0.42 ± 0.09 and 0.76 ± 0.03, respectively, a 1.75-fold increase (n=3, $P < 0.05$). In contrast to previous findings (2), there was no significant difference between the cortical and medullary levels of both isoforms of SOD from high-salt and normal diet fed rats. Cortical Cu/Zn SOD levels from normal and high salt groups were 2.16 ± 0.17 and 2.65 ± 0.48, respectively (n=3). Cortical Mn SOD levels from normal and high salt groups were 1.70 ± 0.11 and 1.69 ± 0.03, respectively (n=3). Medullary Cu/Zn SOD levels from normal and high salt groups were 1.19 ± 0.23 and 1.02 ± 0.07, respectively (n=3). Medullary Mn SOD levels from normal and high salt groups were 1.11 ± 0.01 and 1.03 ± 0.09, respectively (n=3).

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Kitiyakara C, Chabrashvili T, Chen Y, Blau J, Karber A, Aslam S, Welch WJ & Wilcox CS (2003). J Am Soc Nephrol 14, 2775-2782.

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PC16

Prostaglandins E_2 and F_2 decrease vasopressin-induced AQP2 expression in a mouse collecting duct cell line

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Previous studies have speculated that increased prostaglandin (PG) levels seen in several models of nephrogenic diabetes insipidus (NDI) might mediate the decreased aquaporin 2 (AQP2) expression which is thought to underlie the impaired urinary concentrating ability. On the other hand, *in vivo* experiments with non-steroidal antiinflammatory drugs, which inhibit PG synthesis, decreased AQP2 expression, suggesting that PGs may actually increase AQP2 expression (reviewed in Nielsen et al. 2002). These differences may reflect the effects of different prostaglandins and/or receptors in different situations. The mouse collecting duct cell line mpkCCDc14 seems to be the best existing model for the study of the regulation of AQP2 expression in vitro, since it has previously been shown to express the water channel AQP2 in response to vasopressin (VP) stimulation (Hasler et al. 2002) and hypertonicity (Hasler et al. 2005). We have therefore used it to investigate the direct effects of prostaglandins E_2 and F_2 on baseline and vasopressin-induced AQP2 expression.

Cells were grown on Falcon cell culture inserts, until transepithelial resistance (measured using an EVOME voltometer) was

maximal. They were then incubated for 48 h in serum- and hormone-free medium supplemented with 1 nM VP, 100 nM PGE_2, or 100 nM PGF_2, as appropriate: six filters received (i) nothing, (ii) VP, (iii) PGE_2, (iv) PGE_2 + VP, (v) PGF_2, and (vi) PGF_2 + VP. At the end of the incubation period, cells were scraped off the filters and dissolved in Laemmli sample buffer. The experiment was repeated four times, with cells from passages ranging from 25 to 35. Samples were run on 12% polyacrylamide gels, and Western blotted for AQP2, using an affinity purified antibody against the c-terminus of AQP2. Results were analysed using ANOVA, Student's t tests and the false-discovery rate procedure.
In the absence of vasopressin, levels of AQP2 in the cells were undetectable, and treatment with PGE_2 or PGF_2 alone had no detectable effect on this. In contrast, cells treated with VP showed substantial levels of AQP2. Concurrent treatment with PGE_2 decreased AQP2 expression to 43 ± 10% of the levels seen with VP alone, and treatment with PGF_2 reduced it to 57 ± 14% (each $n = 4$, $p < 0.05$).
These results demonstrate that PGE_2 and PGF_2, which are the principal prostaglandins found in the collecting duct, decrease AQP2 expression, at least at this concentration, which is in the high physiological range. Thus increased prostaglandin synthesis, in pathophysiological conditions, may explain the decrease in AQP2 expression seen in a number of forms of acquired NDI.

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PC17

The inhibitory effect of bisphosphonates on calcium oxalate crystal formation *in vitro*

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Calcium oxalate (CaOx) is the most common constituent of urinary stones. These in vitro experiments have studied the physical chemistry of CaOx formation, and analysed the inhibitory effect of bisphosphonates on their aggregation in urine. Calculus growth increases most rapidly by crystal aggregation rather than individual crystal growth; our hypothesis is that stone formation is decreased dramatically if aggregation could be inhibited or even slowed.
CaOx crystals were formed by mixing equal volumes of two solutions, 'A' (containing calcium) and 'B' (containing oxalate). The remaining salts in these solutions combined to form a recognised 'artificial urine' (Robertson & Scurr, 1986). Human urine was not used due to its considerable chemical variability. The solutions were mixed at 37°C and the crystals were formed by the mixed suspension mixed product removal method. The outflow of the mixing chamber was pumped into a Mastersizer (Malvern Instruments, UK) to measure the number and size of particles using low angle laser scattering. Several bisphosphonates (potential inhibitors of crystal aggregation) were added to solution 'B' and the change to the percentage volume occupancy of crystals at sizes 40, 100 and 200 μm was recorded. Data are median values (25%, 75% interquartiles) and changes to volume occupancy from control by bisphosphonates tested by Mann-Whitney U test; significance was accepted at $p<0.05$.
Table 1 shows the volume occupancy of CaOx crystals of different sizes in control conditions and after addition of three different bisphosphonates at 100 and 200 μM. Bisphosphonates 1 and 2 reduced significantly the number of 100 and 200 μm crystals at both concentrations. The third bisphosphonate also reduced the larger crystal numbers at 200 μM.
Bisphosphonates are currently used to treat bone disease and are excreted in the urine. Furthermore, they are resistant to alkaline phosphatase and acid conditions, unlike other inhibitors of urinary crystal aggregation, such as polyphosphates. The ability of these agents to prevent CaOx aggregation to sizes of ≥100 μm will therefore limit their tendency to block nephrons and thus have potential to protect against urinary stone formation.

Table 1. Volume occupancy of CaOx crystals of different sizes in artificial urine containing three different bisphosphonates

	Bisphosphonate concentration (μM)	% Volume of crystal aggregates at the following sizes		
		40μm	100μm	200μm
Control	0	3.32 (2.95,3.55) [6]	2.98 (2.73,5.09) [6]	1.22 (1.08,1.36) [6]
Bisphosphonate 1	100	3.75 (2.91,4.39) [6]	0.24* (0.00,0.65) [6]	0.12* (0.00,0.65) [6]
	200	1.26 (0.99,1.78) [5]	0.00* (0.00,0.00) [5]	0.00* (0.00,0.00) [5]
Bisphosphonate 2	100	3.81 (3.75,4.04) [6]	0.84* (0.28,1.26) [6]	0.45* (0.07,1.26) [6]
	200	2.35 (2.29,2.37) [6]	0.03* (0.00,0.29) [6]	0.00* (0.00,0.29) [6]
Bisphosphonate 3	100	4.60* (4.49,5.04) [4]	0.87* (0.31,1.54) [4]	0.66 (0.48,1.54) [4]
	200	4.87* (4.36,5.82) [4]	0.24* (0.18,0.27) [4]	0.28* (0.08,0.50) [4]

*$p<0.05$ vs control, [number of determinations]

(N.B. Bisphosphonate identities cannot be revealed at this time due to confidentiality agreements).

Robertson WG & Scurr DS (1986). J Urol 135, 1322-1326.

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

PC18

Iminoglycinuria is not caused by mutations in SLC6A20 or SLC36A1

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Specific aminoacidurias have been instrumental in cloning the genes responsible for conditions like cystinuria (basic aminoaciduria) or Hartnup disorder (neutral aminoaciduria).

For iminoglycinuria, candidate genes like SLC6A20 or SLC36A1 have been proposed to be causative. Heterologous expression of these gene's products mediate uptake of proline and glycine (Takanaga et al. 2005; Miyauchi et al. 2005).
We identified two patients with iminoglycinuria by serum and urine amino acid analyses showing typical elevations of fractional excretions of glycine, proline and hydroxyproline only (Coskun et al. 1991).
Sequencing of all coding exons (including splice sites) of SLC6A20 and SLC36A1 showed surprisingly no disease causing mutations.
We conclude that other amino acid transport genes might be responsible for iminoglycinuria.

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PC19

Influence of superoxide anions in the neural control of intra-renal haemodynamics of anaesthetised normotensive and hypertensive rats

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Nitric oxide (NO) and superoxide anions (O2.–) are generated in significant amounts in the kidney, especially in the medulla. The level of O2.- is determined by the activity of superoxide dismutase (SOD) under normal conditions and, due to O2.- degrading NO, this will affect basal levels of renal haemodynamics as well as neurally mediated vasoconstriction. This study investigated whether scavenging of O2.- modulated renal sympathetic nerve (RSN)-induced reductions in renal cortical and medullary blood flow in normotensive and hypertensive rats.
Four groups (n= 9) of male Wistar and stroke prone spontaneously hypertensive rats (SHRSP), 250-300 g, were anaesthetised with 1 ml i.p. chloralose/urethane (16.5/250 mg/ml, respectively). The right femoral vein and artery were cannulated for infusion of saline (154 mM NaCl) at 3 ml/h, anaesthetic supplementation (0.5 ml every 30 min), and measurement of arterial blood pressure (BP). The left kidney was exposed via a flank incision, placed in a holder and a small cannula was inserted 4.5 mm into the kidney for intramedullary (i.m.) infusion of saline or drugs at 0.6-1.0 ml/h. Two Laser-Doppler microprobes were inserted 1.5 and 4.0 mm into the kidney to measure cortical (CP) and medullary (MP) blood perfusion, respectively, in perfusion units (PU). Bipolar stainless steel electrodes were connected to a stimulator and applied to the area in which the renal nerves were usually located. After 90min, baseline values were taken, the stimulation was performed (15V, 2 ms) for 1 min at frequencies of 0.5, 1, 2, 4, 6 and 8 Hz with 5 min rest periods, then either vehicle or tempol (a SOD mimetic) was infused at 30μmol/kg/min i.m. for 90 min and the stimulation protocol was repeated. Data±SEM were subjected to Student's t test and significance taken at $P<0.05$. The animals were killed with an anaesthetic overdose.
In Wistar rats, base line levels of BP, MP and CP were 108±2 mmHg, 78±11 PU and 142±21 PU, respectively, and for SHRSP BP was 130±5 mmHg, MP was 73±9 PU and CP was 112±11 PU. RSN stimulation in the Wistar rats caused a frequency-related reduction in MP and CP of 37% and 74%, respectively (both $P<0.05$), at 8 Hz. After the infusion of tempol MP, but not CP, increased from 108±14 to 125±16 PU ($P<0.05$), the nerve-induced reductions in MP and CP were 30% and 52%, respectively (both $P< 0.05$), the latter being smaller ($P<0.05$) than in the absence of drug. In SHRSP, the RSN stimulation frequency-related decreases in MP and CP were 19% and 51%, respectively (both $P<0.05$) at 8 Hz, and tempol infusion increased MP, but not CP, from 61±8 to 86±10 PU ($P<0.05$), but the magnitudes of the renal nerve-induced responses were unchanged during tempol infusion.
These results indicate that the RSN caused larger reductions in CP than MP in both Wistar and SHRSP, although the responses were larger in the Wistar rats. Scavenging of O2.- with tempol blunted the MP and CP responses, possibly as a result of greater NO availability buffering the neural vasoconstrictions. These potential relationships appeared disturbed in the SHRSP.

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PC20

Protein restriction *in utero* leads to increased salt appetite in the rat

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The spontaneously hypertensive rat has an exaggerated preference for salt solutions, which has led to the suggestion that this model seeks out solutions that provide a volume stimulus to the circulation, contributing to the development of hypertension (1). Rats exposed to protein restriction *in utero* develop hypertension and renal dysfunction (2), but it is not known whether sodium homeostasis is perturbed in this model. Accordingly, the aim of this study was to assess salt appetite, sodium homeostasis and expression of key renal sodium transporters in male rats exposed to protein restriction *in utero*. Female Wistar rats were given a diet containing 18% (control, C) or 9% casein (low protein, LP) from day one of conception until birth. Mothers were then switched to a standard rat chow containing 18% protein; pups were weaned onto this diet. At 4 weeks of age, rats were housed individually and offered a two-bottle choice of either tap water or 0.9% saline for 6 days. Extracellular fluid (ECF) volume was determined in another group of rats. Under Inactin anaesthesia (thiobutabarbital sodium, 100 mg/kg, i.p.) the renal vessels were occluded and ^{3}H inulin was injected (i.v.); serial blood samples were collected to determine the dilution of ^{3}H inulin. A third group of Inactin anaesthetised (100 mg/kg, i.p.) rats were also prepared for a standard clearance study using a servo-controlled fluid replacement system. Saline (0.9%) was infused (i.v.) at a rate matching spontaneous urine output. Kidneys were harvested, post mortem, from a further group of rats for Western blotting and measurement of Na^+-K^+,ATPase activ-

ity to estimate key Na^+ transporter expression and function. Data are presented as mean ± SEM; statistical analysis was by t test. LP rats drank significantly more saline than controls (C 4.8±1.8 vs LP 9.4±1.1 ml (100g bwt)$^{-1}$ 24h^{-1}, P<0.05, n=22-24 per group). ECF volume was significantly larger in LP compared with controls (C 23.5±1.2 vs LP 28.3±1.2 ml (100g bwt)$^{-1}$, n=9-13 per group). Blood pressure, urine flow rate (UV), Na^+ excretion rate ($U_{Na}V$) and fractional excretion of Na^+ (FE_{Na}) were higher in LP rats, but GFR did not differ (Table 1).

Sodium-hydrogen exchanger (NHE3) expression was not altered; however, the sodium-potassium-chloride cotransporter (NKCC2) was upregulated by 135% in the LP group. Na^+-K^+,ATPase activity was significantly reduced in LP rats compared with controls (C 23.4±1.7 vs LP 17.7±1.2 nM PO_4 (g protein)$^{-1}$ h^{-1}, P<0.05, n=10-17 per group). These findings show that sodium homeostasis is disturbed in rats exposed to protein restriction *in utero*. Renal sodium retention does not appear to be the underlying cause of the expanded ECF volume in LP rats. Rather, an increased salt appetite may contribute to the elevation in blood pressure.

	C (*n*=17)	LP (*n*=17)
Mean arterial blood pressure (mmHg)	83±3	105±3*
UV (µl min^{-1} (100g bwt)$^{-1}$)	16.7±3.2	29.6±3.3*
$U_{Na}V$ (µmol min^{-1} (100g bwt)$^{-1}$)	1.4±0.4	3.1±0.4*
FE_{Na} (%)	1.7±0.4	2.9±0.4*

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

PC21

A mathematical model for calculating the risk of crystallisation of calcium salts in the renal tubule

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The aim of this study was to devise a mathematical model to approximate tubular fluid composition in order to calculate the risk of precipitation of Ca salts in the renal tubule between the glomeruli and ducts of Bellini in the human kidney.

A dynamic model of the human kidney was devised to generate tubular fluid composition continuously along the renal tubule. The model included data on pH and the concentrations of Ca, Mg, Na, K, NH^{4+}, P, oxalate (Ox), citrate (Cit), SO_4^{2-} and uric acid (UA) within tubular fluid. The relative supersaturation (RSS) of Ca salts was calculated at all points along the renal tubule using SUPERSAT [1]. The effect of varying the tubular reabsorption of water and of H^+, Ca, P and Ox on RSS was evaluated. In a pilot study, plasma and renal conditions were constructed to yield average urine compositions produced by normal subjects (N) (vol 1.5 litre, pH 5.94, (in mmol/day) Ca 5.4, Mg 4.4, Na 189, K 58, NH^{4+} 23, P 20, Ox 0.33, Cit 2.6, SO_4^{2-} 16 and UA 3.2), recurrent CaOx stone-formers (SF) (vol 1.4 litre, pH 5.94, Ca 8.4, Mg 3.8, Na 189, K 58, NH^{4+} 23, P 29, Ox 0.67, Cit 1.5, SO_4^{2-} 16 and UA 4.5) and recurrent mixed CaOx/CaP SF (vol 1.4 litre, pH 6.55, Ca 8.4, Mg 3.8, Na 189, K 58, NH^{4+} 23, P 29, Ox 0.67, Cit 1.5, SO_4^{2-} 16 and UA 4.5) [2].

A summary of the RSS values of CaOx and CaP at different points along the renal tubule of N and SF is shown in Table 1.

In N, tubular fluid is undersaturated with both CaOx and CaP throughout most of the renal tubule becoming only slightly supersaturated in the loop of Henle and in the late collecting duct. In recurrent Ca SF, it is mildly supersaturated with CaOx in the loop and becomes increasingly supersaturated through the CD. In addition, urine from SF is moderately supersaturated with CaP in the loop and increasingly supersaturated through the CD, particularly in the patients with mixed CaOx/CaP stones.

The model provides a useful tool (a) to predict the risk of crystallisation at all points along the renal tubule, (b) to correlate with patterns of stone-formation and nephrocalcinosis observed *in vivo* and (c) to understand better the underlying pathophysiology of nephrolithiasis.

Table 1. RSS values

Salt	Subjects	Relative supersaturation (RSS)							
		in PT	in DLH	in ALH	in DT	in ECD	in MCD	in LCD	Urine
CaOx	Normals	u	u	u	u	u	u	+	+
	CaOx SF	u	+	+	u	+	++	+++	++++
	CaOx/CaP SF	u	+	+	u	+	++	+++	++++
CaP	Normals	u	+	u	u	u	u	+	+
	CaOx SF	u	++	+	u	u	u	+	+
	CaOx/CaP SF	u	++	+	u	u	++	+++	++++

u, undersaturated; +, weakly supersaturated; ++, moderately supersaturated; +++, highly supersaturated with crystallisation; ++++, grossly supersaturated with marked crystallisation; PT, proximal tubule; DLH, descending limb; ALH, ascending limb; DT, distal tubule; ECD, MCD and LCD, early, mid and late collecting ducts.

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