Purinergic Signalling (2009) 5:481–489
DOI 10.1007/s11302-009-9150-6

REVIEW

ENaC, renal sodium excretion and extracellular ATP

Scott SP Wildman · Esther S-K Kang · Brian F King

Received: 19 July 2007 / Accepted: 15 March 2008 / Published online: 21 March 2009

Abstract Sodium balance determines the extracellular fluid volume and sets arterial blood pressure (BP). Chronically raised BP (hypertension) represents a major health risk in Western societies. The relationship between BP and renal sodium excretion (the pressure/natriuresis relationship) represents the key element in defining the BP homeostatic set point. The renin–angiotensin–aldosterone system (RAAS) makes major adjustments to the rates of renal sodium secretion, but this system works slowly over a period of hours to days. More rapid adjustments can be made by the sympathetic nervous system, although the kidney can function well without sympathetic nerves. Attention has now focussed on regulatory mechanisms within the kidney, including extracellular nucleotides and the P2 receptor system. Here, we discuss how extracellular ATP can control renal sodium excretion by altering the activity of epithelial sodium channels (ENaC) present in the apical membrane of principal cells. There remains considerable controversy over the molecular targets for released ATP, although the $P2Y_2$ receptor has received much attention. We review the available data and reflect on our own findings in which ATP-activated P2Y and P2X receptors make adjustments to ENaC activity and therefore sodium excretion.

Keywords $P2X_4$ receptor · $P2Y_2$ receptor · Amiloride-sensitive currents · ATP · Nucleotide · Kidney · Blood pressure

S. S.P. Wildman (✉)
Department of Veterinary Basic Sciences,
Royal Veterinary College,
Royal College Street,
London NW1 0TU, UK
e-mail: swildman@rvc.ac.uk

E. S.-K. Kang
Laboratory of Molecular Signalling, Babraham Institute,
Babraham,
Cambridge CB2 4AT, UK

B. F. King
Department of Neuroscience, Physiology
and Pharmacology (Royal Free Campus),
University College London,
Rowland Hill Street,
London NW3 2PF, UK

Introduction

Epithelial sodium channels (ENaC) in the apical membrane of kidney cells, in tandem with sodium pumps (Na/K-ATPase) in the basolateral membrane, form an efficient pathway for sodium reclamation in the distal nephron. Adjustments to this salvage pathway come under the control of the renin–angiotensin–aldosterone system (RAAS) and, for many years, researchers working on sodium balance have mainly focussed on this powerful hormonal system. However, within the kidney, a new "intracrine" system has emerged and involves the local release of the energy molecule, ATP, by epithelial cells. This new focus of attention has led us, and others, to propose that ATP-activated P2 receptors (P2Rs) fine tune the activity of ENaC channels in the distal nephron and adjust the rate of sodium excretion by the kidney. Extracellular ATP-based regulation of sodium excretion is not only found in the kidney but also occurs in other hollow organs where sodium ions can be reclaimed (lungs, gastrointestinal tract, pancreatic duct, hepatic and biliary ducts and eccrine sweat ducts). The pharmacological manipulation of P2R populations linked to ENaC may have therapeutic potential in helping to correct a

number of disease states, such as cystic fibrosis [1, 2], polycystic kidney disease [3] (and see article by Turner *et al.* [4] in this Special Issue) and vascular hypertension associated with salt-retention [5–7]. Here, we review this new body of work on P2R/ENaC interactions in renal epithelia and address the role of ATP regulatory mechanisms in controlling the sodium balance in the extracellular fluid.

ENaC in epithelial transport

ENaC comprises three non-identical, yet homologous subunits (α, β and γ subunits) [8]. Each of these structurally different subunits folds and twists in the same way, which results in two hydrophobic regions embedded in the cell membrane and bridged by a large extracellular loop containing >80% of the subunit protein [8]. Relatively short cytoplasmic amino and carboxyl termini project from these two transmembrane spanning regions and extend into the cytoplasm of the epithelial cell [8]. By coincidence, this pattern of folding is also used in processing the protein subunits that form ATP-gated homotrimeric ion channels [9]. ENaC exists as heterotetrameric assemblies, principally involving two α-subunits, one β-subunit and one γ-subunit ($\alpha_2\beta\gamma$)—although other stoichiometries, including homomeric assemblies, do occur, but with lower frequency and function poorly as ion channels [10]. The $\alpha_2\beta\gamma$ assemblies form highly selective and constitutively opened sodium channels which can be blocked by the diuretic compound, amiloride. Amiloride is generally viewed as a specific inhibitor of ENaC, but this drug can also block other membrane transport pathways (including: Na^+/H^+ and Na^+/Ca^{2+} exchangers and the Na^+/K^+ pump) [11]. At low micromolar concentrations, amiloride inhibits ENaC far more effectively than other types of ion channels [11] and, when used at such low concentrations, any observed reduction in membrane current is typically attributed to a reduction in sodium influx through ENaC. Fortunately, amiloride and its related analogues do not block inward sodium currents carried by ATP-gated P2R ion channels [12].

ENaC has a variety of physiological functions. Foremost, it is important in controlling whole-body sodium balance via regulated sodium uptake in the distal nephron [13]. There are secondary roles for ENaC which can modify the volume and composition of secreted fluids. In turn, these adjustments to epithelial secretions can affect ancillary physiological functions such as the rate of mucociliary clearance in the lungs [14]. At these sites and elsewhere, ENaC activity is determined by the rates of channel synthesis, ER-maturation, ER-trafficking, membrane insertion, endocytosis and ion channel open probability [6, 15, 16]. ENaC activity is tightly controlled by hormones (principally aldosterone) but, to varying degrees, it is also modulated by extracellular ATP acting on its surface receptors (P2Rs) [17].

ATP and its surface receptors

Coincident with the molecular isolation of two extended families of surface receptors for extracellular nucleotides, researchers worldwide have expressed a growing interest in extracellular ATP as a physiological regulator of kidney function. ATP, and other signalling nucleotides, are now known to be released from epithelial cells in measured amounts, and in a polarised manner, in response to such diverse stimuli as mechanical stimulation (*e.g.* stretch and osmotic swelling), local acidosis and hypoxia [18–21] (and see article by Praetorius and Leipziger [22] in this Special Issue). The process of ATP release involves a number of complementary pathways that include transport via ATP-binding-cassette (ABC) proteins [23], connexin hemichannels [24], large-diameter anion channels [25] and exocytotic vesicular release [26]. It is now widely accepted that released ATP can act as a local hormone on both sides of the kidney tubule, where this molecule has ample choices for molecular targets. The presence of almost all of the molecularly identified subtypes of ionotropic (P2X) and metabotropic (P2Y) receptors for ATP has been demonstrated in the apical and/or basolateral membranes of native epithelial cells and immortalised epithelial cell lines [7, 17, 27–29]. Ionotropic P2X receptors are represented by homomeric assemblies ($P2X_{1,2,3,4,5,6,7}$) and heteromeric assemblies ($P2X_{1/2}$, $P2X_{1/4}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$, $P2X_{4/7}$). This provides a lengthy list of molecular targets; fortunately, kidney cells are endowed with only a few of these P2X receptor subtypes (primarily $P2X_4$ and $P2X_6$ subunits) and not the entire complement. The localisation of P2X receptor subtypes has been reported as highly regional, occasionally overlapping and sometimes discrete, but never ubiquitous. Metabotropic P2Y receptors are also represented by many of the known subtypes (chiefly $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, but not yet $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$), which again may be found in overlapping regional distributions, but never ubiquitously distributed [7, 17, 28, 29].

ENaC modulation by P2Rs in the kidney

Although the early investigations of ENaC channel modulation/regulation focussed on epithelial cells outside the kidney (see Table 1), most modern studies have shifted attention to renal targets and specifically principal cells (PCs) of the collecting duct (CD) and PC-derived cell lines where ENaC is naturally concentrated. A schematic model summarising all proposed mechanisms of P2R-mediated

Table 1 P2R actions on ENaC

Epithelia	Species	P2R involved (localisation)	Effect on $I_{am\text{-}s}$	Mechanism	Ref. #(s)
Leech integument	*Hirudo medicinalis*	P2X, P2Y (ap., baso.)	Potentiates	–	[54]
Frog skin	*Rana temporaria*	$P2Y_2$ (baso.)	Potentiates	$\uparrow[Ca^{2+}]_{int}$, PKC	[43]
Respiratory epithelium					
Nasal	Human	$P2Y_2$ (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$	[14]
Nasal, bronchial	Rabbit	P2X, P2Y (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$	[55]
Bronchial 1º cultures	Human	$P2Y_2$ (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$	[56]
Trachea	Mouse	$P2Y_2$ (ap.)	Inhibits	PLC, $\downarrow[Cl^-]_{int}$, $\downarrow PIP_2$, MAPK, ↓open prob.	[2, 35, 57–60]
Trachea	Pig	$P2Y_2$ (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$	[61]
Trachea	Rabbit	$P2Y_2$ (ap.)	Inhibits	–	[62]
Distal bronchi 1º cultures	Pig	$P2Y_2$ (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$	[63]
Bronchoalveolar	Mouse	P2Y (ap.)	Inhibits	–	[64]
Alveolar	*Xenopus*	P2X, P2Y (ap.)	Potentiates	–	[65]
Distal foetal lung	Rat	$P2Y_2$ (ap.)	Inhibits	–	[66]
Colon					
Distal colon	Mouse	$P2Y_2$ (ap.)	Inhibits	–	[59, 60, 67]
Distal colon	Guinea-pig	$P2Y_2$ (ap.)	Inhibits	–	[68]
Uterus					
Endometrium	Mouse	$P2Y_4$ (ap.)	Inhibits	$\uparrow[Ca^{2+}]_{int}$, $\downarrow[Cl^-]_{int}$	[69]
Thyroid epithelium	Pig	$P2Y_4$ (ap.)	Inhibits	–	[70]
Kidney					
CCD	Mouse	$P2Y_2$ (ap., baso.)	Inhibits	–	[32]
CD	Rat	$P2X_4$-like (ap.)	Inhibits	–	[49]
CCD/OMCD	Rat	$P2Y_2/P2Y_4$ (ap.)	Inhibits	PKC	[7]
		$P2X_4/P2X_{4/6}$ (ap.)	Inhibits or Potentiates	? or PI3K	[7]
M1 cell line	Mouse	$P2Y_2$ (ap., baso.)	Inhibits	$\uparrow[H^+]_{int}$	[31, 33]
A6 cell line	*Xenopus*	$P2Y_2$ (ap.)	Inhibits	PLC, $\downarrow PIP_2$, ↓open prob.	[34]
		$P2X_4$-like (baso.)	Potentiates	PI3K, ↑open prob.	[45]
CCD 1º cultures	Rabbit	$P2Y_2$-like (ap., baso.)	Inhibits	PLC, PKC	[30]
mIMCD-K2 cell line	Mouse	$P2X_3$, $P2X_4$, $P2Y_1$, $P2Y_2$ (ap.)	Inhibits	–	[37]

A summary of the effects of P2R activation on ENaC activity

$I_{am\text{-}s}$ amiloride-sensitive current (*i.e.* ENaC-mediated current), *ap.*, apical membrane, *baso.* basolateral membrane ↑ increase, ↓ decrease, *PKC* protein kinase C, *PLC* phospholipase C, *PI3K* phosphoinositide 3-kinase, *PIP_2* phosphatidylinositol bisphosphate, *MAPK* mitogen-activated protein kinase, *1°* primary cell cultures, *open prob.* single channel opening probability, *CCD* cortical collecting duct, *OMCD* outer medullary collecting duct

modulation of ENaC in PCs of the CD is shown in Fig. 1, and discussed below.

Koster *et al*. were the first to report that ENaC activity and thus transcellular sodium transport were inhibited by nucleotide activation of renal P2Rs, via a downstream mechanism involving intracellular signalling pathways [30]. Using primary cultures of rabbit CD cells grown to confluence on permeable filters, these investigators dem-

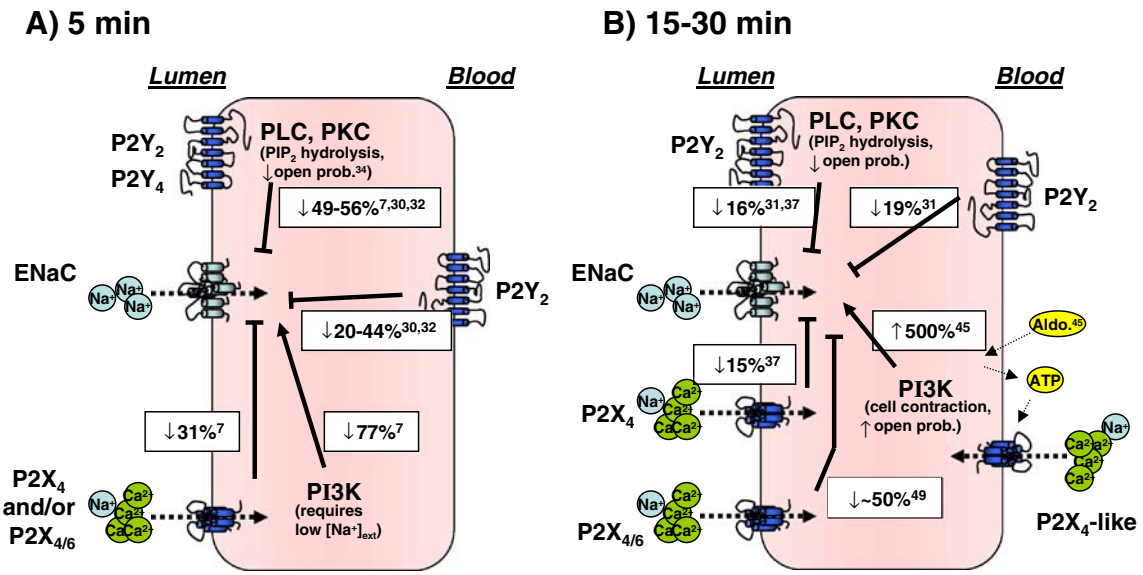

Fig. 1 A summary of known effects of P2R activation on ENaC activity taken from experiments using renal principal cells (PCs) or distal nephron-derived cell lines. Relevant references are superscripted. In **a**, effects where 5 min or less are left between P2R activation and measurements of ENaC activity. All but apical $P2X_{4/6}$ activation decrease the activity of ENaC. Apical $P2X_{4/6}$ has the ability to inhibit or potentiate ENaC activity depending on the concentration of luminal Na^+. Noteworthy is that basolaterally expressed $P2X_4$ receptors have not been reported to affect ENaC activity. In **b**, effects where 15–30 min are left between P2R activation and measurement of ENaC activity. All apically expressed P2Rs inhibit ENaC, although the ability of P2Y receptors to inhibit ENaC is less than that in **a** (from 49–56% to 16%). The potentiating effect of apical $P2X_{4/6}$ receptors (when luminal Na^+ is low) has not been investigated over a 30-min period, but a potentiating effect of basolaterally expressed $P2X_4$-like receptors has been reported

onstrated that either apically or basolaterally applied UTP (100 μM) inhibited amiloride-sensitive sodium transport across cell monolayers. Here, maximal inhibition (56% by apical UTP and 44% by basolateral UTP) occurred within 10 min. The IC_{50} value for UTP inhibition was 500 nM, a concentration that matches the extracellular levels of ATP estimated in tubular fluid [18]. Of note, the proposed P2R subtype responsible for this inhibition was equally sensitive to UTP and ATP. On this basis, inhibition of ENaC was attributed to the activation of metabotropic $P2Y_2$-like receptors. These $P2Y_2$-like receptors coupled downstream to PLC, and their activation by UTP (or ATP) led to a rise in intracellular calcium ($[Ca^{2+}]_{int}$). In turn, raised $[Ca^{2+}]_{int}$ resulted in the activation of PKC which was proposed to mediate the inhibition of ENaC in rabbit CD cells. UTP inhibition of sodium transport was also confirmed in the mouse M1 collecting duct (CD) cell line [31] and in mouse-isolated CDs [32]. Curiously, the early data of Cuffe *et al.* [31] stand apart from other early studies inasmuch as ATP (100 μM) evoked only a modest degree of inhibition (16–19%), probably attributable to the long (30 min) period between P2R activation and assessment of its effects on ENaC activity (see Fig. 1).

The $P2Y_2$-based model of ENaC inhibition proposed by Koster *et al.* [30] has been re-evaluated in an extensive series of derivative studies. Leipziger *et al.*, who used M1 cells, confirmed that $P2Y_2$ receptors do exert an inhibitory effect on ENaC, but notably without the involvement of PKC [33]. Ma *et al.* next showed that evoked release of extracellular ATP from the apical membrane of *Xenopus*-derived A6 distal nephron cells in response to cell stretch (of itself, an interesting observation) inhibited sodium transport principally through a PLC-dependent pathway that decreased the open probability of ENaC [34]. Here, it was proposed that PLC activation reduces the PIP_2 concentration in the inner face of the cell membrane and that membrane PIP_2 normally binds to the N-terminus of the β-ENaC subunit to increase the open probability of ENaC [35]. Accordingly, ENaC activity falls when $P2Y_2$ receptors are stimulated by extracellular UTP and when PIP_2 is scavenged by PLC to form soluble IP_3 and DAG. Unsurprisingly, $P2Y_2^{-/-}$ gene deletion results in a facilitated Na^+ reabsorption in the kidney but, unexpectedly, it mainly involves a novel compensatory mechanism that increases the expression of Na–K–2Cl cotransporters in mouse thick ascending limb (TAL) epithelial cells, with only a marginal facilitation of ENaC activity and no significant change in ENaC number [36]. In some ways, such latent compensatory mechanisms have hindered progress in understanding P2R function in the kidney.

The work of McCoy *et al.* next stands out in the debate of ENaC regulation by extracellular ATP. These investigators were the first to propose that ENaC inhibition involved not only P2Y receptors but also P2X receptors,

in the immortalised K2-cell line from mouse inner medullary CD (mIMCD) [37]. Here, ATP or UTP (100 μM) caused only a modest reduction (~15%). However, ENaC inhibition was just as pronounced following P2XR activation with αβmeATP as by P2YR activation with UTP and, of note, only apically applied nucleotides inhibited ENaC activity. On the basis of RT-PCR data, McCoy *et al*. further suggested that ENaC inhibition in mIMCD-K2 cells was via $P2X_3$ and $P2X_4$ receptor subtypes expressed on the apical membrane. The activation of epithelial P2X receptors not only inhibited ENaC but also activated chloride currents—a phenomenon better associated with P2Y receptor function in epithelial studies. However, epithelial P2X receptors can generate chloride currents in several ways. For example, calcium influx through P2X receptors can lead to the activation of Ca^{2+}-dependent chloride currents, which can be prevented either by substituting extracellular calcium with equimolar barium or by adding chloride channel blockers (such as DIDS or NPPB) to the bathing medium. Additionally, some P2X receptors (notably, human $P2X_5$) lose their selectivity for cations with time and become increasingly permeable to chloride [38].

Using a split-tubule preparation of rat distal CDs, our own experiments reveal that PCs possess both apical P2Y and P2X receptors, and that each receptor subclass regulates ENaC activity [7]. We have located at least two types of P2Y receptor ($P2Y_2$ and $P2Y_4$ subtypes) on the apical border, identified immunohistochemically in the cell membrane and by real time-PCR within the CD. In rodents, these two P2Y subtypes are notoriously difficult to distinguish pharmacologically because their profiles are so alike and differ only in minor ways [39]. Activation of this mixed P2Y receptor population with 10 μM UTP inhibited ENaC activity by ~50% within 3 min, by an effect involving a PLC-dependent pathway and inhibited by U73122. $P2X_4$ and $P2X_6$ subunits are also present in the apical membrane of rat CD; again, their proteins were localised by immunohistochemistry and their transcripts identified by real time-PCR. Depending on the concentration of luminal sodium, activation of $P2X_4$ receptors (and possibly $P2X_{4/6}$ receptor complexes) with 2meSATP (10 μM) either inhibited or potentiated ENaC activity. When luminal sodium was low (50 mM; which is considered "normal" for the CD), ENaC activity was potentiated by 80% (within 3 min) after P2X receptor activation, and this effect involved a PI3K-dependent pathway which was inhibited by wortmannin. When luminal sodium was high (145 mM; which is the concentration typically used in electrophysiology experiments), ENaC activity was inhibited by 30% when P2X receptors were activated. These findings have led us to propose that $P2X_4$ and $P2X_{4/6}$ receptors might act as apically expressed sodium sensors for the rapid and local regulation of ENaC activity in the rat CD [7]. Additionally, these findings show that close attention must be paid to the composition of bathing fluids, which should be biased towards the composition of the luminal fluid in the distal tubule rather than typical representations of extracellular fluid, such as Ringer's, Tyrodes or Krebs solution.

In earlier experiments, we had shown that a single (once-only) activation of $P2X_4$ and $P2X_{4/6}$ receptors resulted in the persistent inhibition of sodium influx in cells co-expressing ENaC [40]. We had failed to observe any potentiation of recombinant ENaC activity, but we did not reduce extracellular sodium to low levels (50 mM). However, it is notable that the probability of channel opening and the magnitude of inward currents carried by $P2X_4$-like receptors in airway epithelia can be enhanced when extracellular sodium concentrations are very low [41, 42]. How such enhancements of $P2X_4$-like receptor function might lead to a potentiation of renal ENaC activity is still to be resolved, but a hint comes from a series of interconnected findings. First, McCoy *et al*. reported that ATP responses in mIMCD-K2 cells were biphasic and comprised an initial reduction in sodium transport, followed by a small secondary rise [37]. Second, similar biphasic responses to serosally applied ATP were observed in frog skin epithelium, where the initial reduction in sodium transport occurred at the same time as a transient elevation in intracellular calcium, but the secondary rise in sodium transport was associated with the apical insertion of new sodium channels [43]. The initial reduction in ENaC activity was attributed to the activation of a P2X receptor and, subsequently, a $P2X_5$-like receptor was cloned from frog skin [44]. These findings are put into perspective by the final observation that activation of a basolateral P2X receptor in *Xenopus* renal A6 epithelial cells alters cell shape by a rearrangement of the cytoskeleton, which results in increased sodium transport [45] brought about by the unruffling of the apical membrane and insertion of ENaC [46]. The basolateral P2X receptors in renal A6 cells were identified pharmacologically as $P2X_4$-like [45], although their precise molecular identity could conceivably involve heteromeric assemblies of $P2X_4$-like and $P2X_5$-like subunits—to better fit with the above findings in frog skin [44]. Whatever the final identity, a causal link has been established between P2X activation, the apical insertion of ENaC and enhancement of sodium transport. This link might have some bearing on our findings that activation of apical P2X receptors under conditions of low extracellular sodium can potentiate sodium uptake and transport.

The apical stimulation of P2X and P2Y receptors upon reduction of extracellular sodium cannot be addressed in the renal A6 cell model, because this cell membrane is water-impermeable and the cells do not swell in response to

apical perfusion of hypo-osmotic solutions [47]. However, this system remains noteworthy because it shows a robust response to changes in tonicity at the basolateral membrane. Here, hypo-osmotic solutions (140 mOsmol; Na^+, 70 mM) evoke a pronounced biphasic pattern of ATP release [48]. The first phase was transient and occurred with cell swelling and was replaced by a later tonic phase of sustained ATP release. This first phase of ATP release coincided with a transient increase in $[Ca^{2+}]_{int}$ which, of itself, was inhibited by suramin and suggested that suramin-sensitive P2Rs were involved. The magnitude of the increase in $[Ca^{2+}]_{int}$ also correlated linearly with the rate of ATP release and, surprisingly, a reciprocal correlation between the concentration of extracellular ATP ($[ATP]_{ext}$) and $[Ca^{2+}]_{int}$ was noted. Thus, a feed-forward system may occur in renal A6 cells, which is triggered by basolateral hypotonicity and causes a surge of ATP release. In turn, ATP stimulates suramin-sensitive P2Rs to increase $[Ca^{2+}]_{int}$ and evoke further ATP release. It was proposed that an ATP plateau was reached only when the rate of ATP release was balanced by the rate of ATP consumption by ectoATPases [48].

The presence of a similar feed-forward system in the apical membrane of other CD-like cell lines, or the intact CD itself, would have interesting consequences for the regulation of sodium transport. This topic was addressed by microperfusion studies of rat CD, *in vivo* [49]. In rats maintained on a low sodium diet to enhance ENaC activity, late distal tubular perfusion of the stable analogue ATPγS significantly increased ^{22}Na recovery in the urine (measured after 15 min), by a calculated 50% reduction in ENaC-mediated sodium reabsorption—based on corresponding reductions evoked by perfused amiloride. Tubular perfusion of the P2Y receptor stimulants Ap_4A, Cp_4U and BzATP had no significant effect on ^{22}Na recovery, suggesting that $P2Y_2$ and $P2Y_4$ receptors were not primarily involved. However, recombinant $P2X_4$ and $P2X_{4/6}$ receptors can be activated by ATPγS, but not by Ap_4A, nor Cp_4U, and only weakly by BzATP [50]. These complementary experiments indicate that the apical $P2X_4$-like receptors identified in rat CD [7] might represent the most likely molecular target for ATPγS perfusions *in vivo*. A debatable point to this *in vivo* study was the use of high ATPγS concentrations (1 mM) to elicit changes in ^{22}Na recovery. However, each CD receives luminal fluid from up to ten distal tubules and, by a tenfold dilution, the maximal concentration would approximate ~100 μM, a concentration sufficient to maximally stimulate $P2X_4$ receptors at pH 7.4 but below the maximum effective concentration if luminal fluid was acidic (<pH 7.0) [51]. Of note, the maximum effective concentration to stimulate P2X receptors in principal cells of the split-tubule rat CD was ~100 μM (pH 7.4 and Na^+ 50 mM) [7].

P2 receptor density dependent on ENaC expression

While much of this review has focussed on the role of P2Rs in adjusting the activity of ENaC, evidence is gradually accruing for a reciprocal arrangement whereby ENaC affects the membrane density of P2Rs. Holbird *et al*. first demonstrated that aldosterone treatment (~60 h) upregulated P2X receptor-evoked short circuit currents across the bullfrog skin—a model where $P2X_5$-like receptors can either inhibit or potentiate ENaC activity and sodium transport [52]. In a similar vein, we have demonstrated that mRNA levels for $P2X_4$ and $P2X_6$ receptors; and for the $P2Y_4$ receptor, are markedly elevated in PCs of rat CD [7] in animals maintained on a low sodium diet for 10 days to elevate the circulatory levels of aldosterone and ENaC expression. Using the *Xenopus* oocyte expression system, we have also shown that the maximal currents evoked by stimulating different P2X assemblies ($P2X_2$, $P2X_{2/6}$, $P2X_5$) were significantly increased if these P2X receptors were co-expressed with functional ENaC [40]. This increased functionality was attributed to an increased P2X receptor density in the cell membrane, with larger amounts of detectable P2X protein found in membrane fractions of cell homogenates. This observation suggested that ENaC either affected the rates of P2X receptor insertion into, or restricted the rates of P2X receptor retrieval from, the cell membrane. The outcome of experiments using Brefeldin A to inhibit the insertion of new P2X receptors pointed to a reduced retrieval of P2X receptors as the likely cause of ENaC-induced upregulation of P2X receptor function. Based on these observations, we have postulated that protein–protein interactions may occur between P2X and ENaC assemblies to delay P2X receptor internalisation. This likelihood is supported by already-proven examples of intracellular cross-talk and physical interactions between P2X receptors and other ligand-gated ion channels such as serotonin-gated $5\text{-}HT_3$ receptors, acetylcholine-gated nAChR complexes ($\alpha_4\beta_2$) and $GABA_C$ complexes [53].

Conclusions

The internal renal mechanisms that help to control sodium uptake from the distal nephron and sodium loss into the urine are complex and still poorly understood. However, inroads have been made by investigators in number of laboratories towards understanding the role of extracellular ATP in this process.

We have used expressions systems to study many types of P2Rs in isolation and, latterly, co-expressed these P2Rs with ENaC. Next, our data from molecular modelling of P2R/ENaC interactions have been tested against data from split-tubule work on rat CDs. Thus far, we have avoided the

use of CD-derived cell lines although we do acknowledge that there is considerable value to their use. We have carried out extensive surveys of the distribution of P2Rs along the nephron, cross-correlated with the presence of transcripts for P2X and P2Y receptor subtypes in PCs. Additionally, our colleagues have worked with us on related studies of single microperfused renal tubules, *in vivo*.

Our developing understanding of this internal renal system is that ATP released from either the apical membrane alone, or from the basolateral border alone, cannot conveniently explain how ENaC activity is regulated. It requires polarised ATP transport on both sides of the epithelial cell, where there are several key molecular targets for this released nucleotide. The initial focus on $P2Y_2$ receptors must be carefully reassessed, partly because it is still inordinately difficult to distinguish the rat isoform of the $P2Y_2$ subtype from the $P2Y_4$. Both $P2Y_2$ and $P2Y_4$ receptors are expressed in PCs in the CD, and separating their functions will probably require the development of selective agonists and antagonists.

In addition to these P2Y subtypes, P2X receptors make a contribution to ENaC turnover and activity. Here, the problem becomes more confusing because the efficiency of P2X receptors as ion channels is highly dependent on extracellular levels of H^+ and Na^+ ions. Scant attention has been paid to the composition of luminal fluid as a determinant of P2R function. The $P2X_4$ receptor is emerging as a key player in the control of ENaC. This P2X subtype is also difficult to manipulate, with limited pharmacological tools available and little understanding of its operational profile *in vivo*. Additionally, the $P2X_4$ subunit may polymerise with the $P2X_6$ subunit also found in kidney cells, to generate a new type of P2X receptor with divergent properties.

To fully understand this intrarenal and autocrine/paracrine system, it is important to study how the RAAS system affects the expression of P2X and P2Y receptor subtypes as well as ENaC activity and how ENaC itself interacts with these signalling proteins. Preliminary data show that P2R and ENaC do not ignore each other, but can alter the fate of the other. Understanding the cellular mechanisms through which cross-talk occurs is a major undertaking.

A major challenge for the future is understanding how the ATP-based "intracrine" system of the kidney affects long-term BP and the aetiology of hypertension. On a superficial level, hypertension is a noted feature of $P2Y_2^{-/-}$, $P2Y_4^{-/-}$ and $P2X_4^{-/-}$ gene knockout mice although the underlying causes need not necessarily rest with the kidney. Also, the incidence of polymorphisms in the P2Y subtypes and occurrence of alternatively spliced $P2X_4$ and $P2X_6$ transcripts may yet feature in this debate. All these possibilities provide fertile ground for a field of investigation that has been barely explored. We look forward to updating this review in the near future, as these questions and others are addressed by the research community.

Acknowledgements We are grateful for the financial support of the British Heart Foundation and the St Peter's Trust for Kidney, Bladder and Prostate Research. Ms Kang was supported by The Nuffield Foundation.

References

1. Mall M, Bleich M, Greger R, Schreiber R, Kunzelmann K (1998) The amiloride-inhibitable Na^+ conductance is reduced by the cystic fibrosis transmembrane conductance regulator in normal but not in cystic fibrosis airways. J Clin Invest 102:15–21
2. Kunzelmann K, Scheidt K, Scharf B, Ousingsawat J, Schreiber R, Wainwright B, McMorran B (2006) Flagellin of *Pseudomonas aeruginosa* inhibits Na^+ transport in airway epithelia. FASAB J 20:545–546
3. Schwiebert EM, Wallace DP, Braunstein GM, King SR, Peti-Peterdi J, Hanaoka K, Guggino WB, Guay-Woodford LM, Bell PD, Sullivan LP, Grantham JJ, Taylor AL (2002) Autocrine extracellular purinergic signalling in epithelial cells derived from polycystic kidneys. Am J Physiol Renal Physiol 282(4): F763–F775
4. Turner CM, Elliott JI, Tam FWK (2009) P2 receptors in renal pathophysiology. Pur Sig. doi:10.1007/s11302-009-9153-3
5. Guyton AC (1991) Blood pressure control—special role of the kidneys and body fluids. Science 252:1813–1816
6. Ma H-P, Chou C-F, Wei S-P, Eaton DC (2007) Regulation of the epithelial sodium channel by phosphatidylinositides: experiments, implications, and speculations. Pflügers Arch 455:169–80
7. Wildman SS, Marks J, Turner CM, Yew-Booth L, Peppiatt-Wildman CM, King BF, Shirley DG, Wang W-H, Unwin RJ (2008) Sodium-dependent regulation of renal amiloride-sensitive currents by apical P2 receptors. J Am Soc Nephrol 19:731–42
8. Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC (1994) Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. Nature 12:463–467
9. King BF, Townsend-Nicholson A (2003) Nucleotide and nucleoside receptors. Tocris Reviews 23:1–11
10. Firsov D, Schild L, Gautchi I, Merillat A-M, Schneeberger E, Rossier BC (1996) Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. Proc Nat Acad Sci USA 93:15370–15375
11. Kleyman TR, Cragoe EJ (1988) Amiloride and its analogs as tools in the study of ion transport. J Memb Biol 105:1–21
12. King BF, Wildman SS, Ziganshina LE, Pintor J, Burnstock G (1997) Effects of extracellular pH on agonism and antagonism at a recombinant $P2X_2$ receptor. Br J Pharmacol 121:1445–1453
13. Schafer JA (2002) Abnormal regulation of ENaC: syndromes of salt retention and salt wasting by the collecting duct. Am J Physiol Renal Physiol 283:F221–F235
14. Mall M, Wissner A, Gonska T, Calenborn D, Keuhr J, Brandis M (2000) Inhibition of amiloride-sensitive epithelial Na^+ absorption by extracellular nucleotides in human normal and cystic fibrosis airways. Am J Respir Cell Mol Biol 23:755–761
15. Snyder PM (2005) Regulation of epithelial Na^+ channel trafficking. Endocrinology 146:5079–5085
16. Pochynyuk O, Tong Q, Staruschenko A, Ma HP, Stockand JD (2006) Regulation of the epithelial Na^+ channel (ENaC) by phosphatidylinositides. Am J Physiol Renal Physiol 290:F949–F957

17. Unwin RJ, Bailey MA, Burnstock G (2003) Purinergic signalling along the renal tubule: current state of play. News Physiol Sci 18:237–241
18. Schwiebert EM, Kishore BK (2001) Extracellular nucleotide signalling along the renal epithelium. Am J Physiol Renal Physiol 280:F945–F963
19. Schwiebert E, Zsembery A (2003) Extracellular ATP as a signalling molecule for epithelial cells. Biochim et Biophys Acta 1615:7–32
20. Lazarowski ER, Boucher RC, Harden TK (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y- receptor activating molecules. Mol Pharmacol 64:785–795
21. Praetorius H, Frokiaer J, Leipziger J (2005) Transepithelial pressure pulses induce nucleotide release in polarized MDCK cells. Am J Physiol Renal Physiol 288:F133–F141
22. Praetorius HA, Leipziger J (2009) ATP release from non-excitable cells. Pur Sig. doi:10.1007/s11302-009-9146-2
23. Cantiello HF (2001) Electrodiffusional ATP movement through CFTR and other ABC transporters. Pflügers Arch 443:S22–S27
24. Bahima L, Aleu J, Elias M, Martin-Satue M, Muhaisen A, Blasi J, Marsal J, Solsona J (2006) Endogenous hemichannels play a role in the release of ATP from *Xenopus* oocytes. J Cell Physiol 206:95–102
25. Bell PD, Lapointe JY, Sabirov R, Hayashi S, Peti-Peterdi J, Manabe K, Kovacs G, Okada Y (2003) Macula densa cell signalling involves ATP release through a maxi anion channel. PNAS 100:4322–4327
26. Vekaria RM (2004) Vesicular storage and release of ATP in a rat proximal tubule cell line. J Physiol 560P:C17
27. Taylor A, Schwiebert L, Smith J, King C, Jones J, Sorscher E, Schwiebert E (1999) Epithelial P2X purinergic receptor channel expression and function. J Clin Invest 104:875–884
28. Leipziger J (2003) Control of epithelial transport via luminal P2 receptors. Am J Physiol Renal Physiol 284:F419–F432
29. Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ (2003) The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: an immunohistochemistry study. Cells Tissues Organs 175:105–117
30. Koster H, Hartog A, Van Os C, Bindels R (1996) Inhibition of Na^+ and Ca^{2+} reabsorption by P2u purinoceptors requires PKC but not Ca^{2+} signalling. Am J Physiol 270:F53–F60
31. Cuffe J, Bielfeld-Ackermann A, Thomas J, Leipziger J, Korbmacher C (2000) ATP stimulates Cl^- secretion and reduces amiloride-sensitive Na^+ absorption in M-1 mouse cortical collecting duct cells. J Physiol 524:77–90
32. Lehrmann H, Thomas J, Kim SJ, Jacobi C, Leipziger J (2002) Luminal $P2Y_2$ receptor-mediated inhibition of Na^+ absorption in isolated perfused mouse CCD. J Am Soc Nephrol 13:10–18
33. Thomas J, Deetjen P, Ko W-H, Jacobi C, Leipziger J (2001) $P2Y_2$ receptor-mediated inhibition of amiloride-sensitive short circuit current in M-1 mouse cortical collecting duct cells. J Membrane Biol 183:115–124
34. Ma H-P, Li L, Zhou Z-H, Eaton DC, Warnock DG (2002) ATP masks stretch activation of epithelial sodium channels in A6 distal nephron cells. Am J Physiol Renal Physiol 282:F501–F505
35. Kunzelmann K, Bachhuber T, Regeer R, Markovich D, Sun J, Schreiber R (2005) Purinergic inhibition of the epithelial Na^+ transport via hydrolysis of PIP_2. FASEB J 19:142–3
36. Rieg T, Bundey RA, Chen Y, Deschenes G, Junger W, Insel PA, Vallon V (2007) Mice lacking $P2Y_2$ receptors have salt-resistant hypertension and facilitated renal Na^+ and water reabsorption. FASEB J 21:3717–3726
37. McCoy D, Taylor A, Kudlow B, Karlson K, Slattery M, Schwiebert L, Schwiebert E, Stanton B (1999) Nucleotides regulate NaCl transport in mIMCD-K2 cells via P2X and P2Y purinergic receptors. Am J Physiol Renal Physiol 277:F552–F559
38. Bo X, Jiang LH, Wilson HL, Kim M, Burnstock G, Surprenant A, North RA (2003) Pharmacological and biophysical properties of the human $P2X_5$ receptor. Mol Pharmacol 63:1407–1416
39. Wildman SS, Unwin RJ, King BF (2003) Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. Brit J Pharmacol 140:1177–1186
40. Wildman SS, Marks J, Churchill LJ, Peppiatt CM, Chraibi A, Shirley DG, Horisberger JD, King BF, Unwin RJ (2005) Regulatory interdependence of cloned epithelial Na^+ channels and P2X receptors. J Am Soc Nephrol. 16:2586–2597
41. Ma W, Korngreen A, Uzlaner N, Priel Z, Silberberg SD (1999) Extracellular sodium regulates airway ciliary motility by inhibiting a P2X receptor. Nature 400:894–897
42. Ma W, Korngreen A, Weil S, Cohen EB-T, Priel A, Kuzin L, Silberberg SD (2006) Pore properties and pharmacological features of the P2X receptor channel in airway ciliated cells. J Physiol 571:503–517
43. Brodin B, Nielsen R (2000) Electrophysiological evidence for an ATP-gated ion channel in the principal cells of the frog skin epithelium. Pflügers Arch 439:227–233
44. Jensik PJ, Holbird D, Collard MW, Cox TC (2001) Cloning and characterisation of a functional P2X receptor from larval bullfrog skin. Am J Physiol Cell Physiol 281:C954–C962
45. Zhang Y, Sanchez D, Gorelik J, Klenerman D, Lab M, Edwards C, Korchev Y (2007) Basolateral $P2X_4$-like receptors regulate the extracellular ATP-stimulated epithelial Na^+ channel activity in renal epithelia. Am J Physiol Renal Physiol 292:F1734–F1740
46. Gorelik J, Zhang Y, Sanchez D, Shevchuk A, Frolenkov G, Lab M, Klenerman D, Edwards C, Korchev Y (2005) Aldosterone acts via an ATP autocrine/paracrine system: the Edelman ATP hypothesis revisited. PNAS 102:15000–15005
47. De Smet P, Simaels J, Van Driessche W (1995) Regulatory volume decrease in a renal distal tubular cell line (A6). I. Role of K^+ and Cl^-. Pflügers Arch 430:936–944
48. Jans D, Srinivas SP, Waelkens E, Segal A, Lariviere E, Simeals J, Van Driessche W (2002) Hypotonic treatment evokes biphasic ATP release across the basolateral membrane of cultured renal epithelia (A6). J Physiol 545:543–555
49. Shirley DG, Bailey MA, Unwin RJ (2005) *In vivo* stimulation of apical P2 receptors in collecting ducts: evidence for inhibition of sodium reabsorption. Am J Physiol Renal Physiol 288:F1243–F1248
50. Wildman SS, Shirley DG, King BF, Unwin RJ. Indirect evidence for regulation of sodium reabsorption in sodium-restricted rats by an ATP-gated heteromeric P2X receptor. J Physiol 2004: 560P PC11.
51. Wildman SS, King BF, Burnstock G (1999) Modulation of ATP-responses at recombinant $rP2X_4$ receptors by extracellular pH and zinc. Br J Pharmacol 126:762–768
52. Holbird D, Jensik P, Cox T (2001) Aldosterone upregulates purinergic responses in larval amphibian skin epithelium. J Comp Physiol 171:413–420
53. Nicke A, King BF (2006) Heteromerization of P2X receptors. In: Arias HR (ed) *Biological and biophysical aspects of ligand-gated ion channel receptor superfamilies*, Ch 15. Research SignPost, India, pp 383–417
54. Schnizler M, Buss M, Clauss W (2002) Effects of extracellular purines on ion transport across the integument of Hirudo medicinalis. J Exp Biol 205:2705–13
55. Poulsen AN, Klausen TL, Pedersen PS, Willumsen NJ, Frederiksen O (2005) Regulation of ion transport via apical purinergic receptors in intact rabbit airway epithelium. Pflügers Arch 450:227–35
56. Devor D, Pilewski J (1999) UTP inhibits Na^+ absorption in wild-type and ΔF508 CFTR-expressing human bronchial epithelia. Am J Physiol Cell Physiol 45:C827–C837

57. Kunzelmann K, Schreiber R, Boucherot A (2001) Mechanisms of the inhibition of epithelial Na^+ channels by CFTR and purinergic stimulation. Kid Int 60:455–461
58. Kunzelmann K, Scheiber R, Cook D (2002) Mechanisms for the inhibition of amiloride-sensitive Na^+ absorption by extracellular nucleotides in mouse trachea. Pflügers Arch 444:220–226
59. König J, Schreiber R, Mall M, Kunzelmann K (2002) No evidence for inhibition of ENaC through CFTR-mediated release of ATP. Biochim Biophys Acta 1565:17–28
60. Schreiber R, König J, Sun J, Markovich D, Kunzelmann K (2003) Effects of purinergic stimulation, CFTR and osmotic stress on amiloride-sensitive Na^+ transport in epithelia and *Xenopus* oocytes. J Membrane Biol 192:101–110
61. Inglis S, Olver R, Wilson S (2000) Differential effects of UTP and ATP on ion transport in porcine tracheal epithelium. Brit J Pharmacol 130:367–374
62. Iwase N, Sasaki T, Shimura S, Yamamoto M, Suzuki S, Shirato K (1997) ATP-induced Cl^- secretion with suppressed Na^+ absorption in rabbit tracheal epithelium. Resp Physiol 107:173–180
63. Inglis S, Collett A, McAlroy H, Wilson S, Oliver R (1999) Effect of luminal nucleotides on Cl^- secretion and Na^+ absorption in distal bronchi. Pflügers Arch 438:621–627
64. Davis IC, Sullender WM, Hickman-Davis JM, Lindsey JR, Matalon S (2004) Nucleotide-mediated inhibition of alveolar fluid clearance in BALB/c mice after respiratory syncytial virus infection. Am J Physiol Lung Cell Mol Physiol 286: L112–20
65. Fronius M, Berk A, Clauss W, Schnizler M (2004) Ion transport across *Xenopus* alveolar epithelium is regulated by extracellular ATP, UTP and adenosine. Resp Physiol Neurobiol 139:133–144
66. Ramminger SJ, Collett A, Baines DL, Murphie H, McAlroy HL, Olver RE, Inglis SK, Wilson SM (1999) $P2Y_2$ receptor-mediated inhibition of ion transport in distal lung epithelial cells. Br J Pharmacol 128:293–300
67. Matos JE, Sorensen MV, Geyti CS, Robaye B, Boeynaems JM, Leipziger J (2007) Distal colonic Na^+ absorption inhibited by luminal $P2Y_2$ receptors. Pflügers Arch 454:977–987
68. Yamamoto T, Suzuki Y (2002) Role of luminal ATP in regulating electrogenic Na^+ absorption in guinea pig distal colon. Am J Physiol Gastrointest Liver Physiol 283:G300–G308
69. Wang XF, Chan HC (2000) Adenosine triphosphate induces inhibition of Na^+ absorption in mouse endometrial epithelium: a Ca^{2+}-dependent mechanism. Biol Reprod 63:1918–1924
70. Bourke J, Abel K, Huxham G, Cooper V, Manley S (1999) UTP-preferring P2 receptor mediates inhibition of sodium transport in porcine thyroid epithelial cells. Br J Pharmacol 127:1787–92