Basolateral P2X₄-like receptors regulate the extracellular ATP-stimulated epithelial Na⁺ channel activity in renal epithelia

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The potent P2X4 receptor agonist 2-methylthio-ATP (2MeSATP) to action on the epithelial cell basolateral plasma membrane. Addition of resistance measurements, and scanning ion conductance microscopy, lates sodium reabsorption. Using A6 cells, transepithelial electrical dent on the location of these receptors, ATP either inhibits or stimulates sodium reabsorption. Using A6 cells, transepithelial electrical resistance measurements, and scanning ion conductance microscopy, we have identified the purinergic receptors involved in the stimulatory action on the epithelial cell basolateral plasma membrane. Addition of the potent P2X₄ receptor agonist 2-methylthio-ATP (2MeSATP) to the basolateral side of the A6 monolayer stimulated amiloride-sensitive sodium conductance and produced similar cell morphological changes to those found with ATPγS, aldosterone, or hypotonic stress. The agonist potency order determined by sodium conductance changes of the monolayer was: 2MeSATP ≈ ATPγS > CTP, a similar agonist potency profile to that of cloned P2X₄ receptors but with higher sensitivity for β, γ-methylene-ATP and α,β-methylene-ATP. We further demonstrated that the ATP effect on sodium transport was potentiated by ivermectin, not blocked by suramin and PPADS, enhanced by Zn²⁺ but not by Cu²⁺, and significantly reduced but not totally inhibited by brilliant blue G. These results led us to conclude that basolateral P2X₄-like receptors were involved. We suggest that there is a reciprocal purinergic system acting both at a basolateral and apical location for control of Na⁺ transport. This requires a mechanism within the cell that leads to either basolateral or apical ATP release to regulate renal tubular function.

ENaC; scanning probe microscopy; scanning ion conductance microscopy; aldosterone; transepithelial electrical resistance

EXTRACELLULAR ATP ACTS AS an autocrine and paracrine signaling molecule that initiates potent effects on sodium transport across renal epithelia through their membrane-associated purinergic (P2) receptors (6, 30). Dependent on the location of these receptors, ATP has been found to either inhibit or stimulate sodium reabsorption. Those P2 receptors on the apical plasma membrane inhibit Na⁺ reabsorption in the kidney distal tubule (9, 22, 35, 39). In contrast our group has recently observed that basolaterally released ATP stimulates

the opening of amiloride-sensitive epithelial Na⁺ channels (ENaCs) and increases Na⁺ transport in A6 cells (11). These renal epithelial cells are derived from Xenopus laevis and exhibit sodium transport properties similar to the mammalian distal tubule (38). We have now carried out experiments to further identify the purinergic receptors involved in this extracellular ATP-induced stimulatory action.

Receptors for extracellular ATP have been classified into two groups: P2X (ionotropic) and P2Y (G protein coupled) (7, 26). Molecular pharmacological and cloning techniques have revealed the existence of seven P2X subunits, which form seven trimeric nucleotide-gated homomeric ion channels (P2X₁–₇) and five established heteromeric ion channel assemblies (P2X₂/₁/₅, 2/₃/₂₆, 4/₆/₆) (26, 39). We have found using A6 cells and scanning ion conductance microscopy (SICM) that aldosterone or basolateral hypotonic stress stimulates ATP release (11). This autocrine/paracrine ATP release produces progressive cell contraction and apical membrane elevation. Sodium transport closely correlates with the cell contraction and morphological changes. We also found that the general P2 receptor antagonists suramin and/or pyridoxal phosphate-6-azo (benzene-2,4-disulfonic) acid (PPADS) not only did not reduce but suramin markedly increased ENaC activity. This suggested that the effect may be mediated via P2X₄ receptors, which have been demonstrated to be insensitive to these two antagonists (5, 26, 29).

Multiple P2X receptors (P2X₄, P2X₅, and P2X₆) have been immunolocalized to the distal tubule and collecting duct principal cells (39). Using immunohistological techniques and polyclonal antibodies, Unwin and Burnstock’s (38) group has further identified that only P2X₄ and P2X₆ receptors are present on the basolateral membrane of the rat distal tubule epithelium. Despite the detection of these P2X subunits, their physiological function remains unclear. However, such predominant expression of P2X₄ receptors located exclusively on renal tubule cells (6, 38) suggests that they may play an important role in renal function. Given the importance of aldosterone in the control of sodium reabsorption in the distal nephron and our results indicating that aldosterone stimulates sodium transport via an ATP-autocrine/paracrine system (11), we have now further investigated the involvement of P2X₄-like receptors in the extracellular ATP-stimulated epithelial sodium transport across renal epithelia.

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MATERIALS AND METHODS

Cell culture. A single X. laevis renal epithelial A6 cell line (kindly provided by Dr. P. DeSmert, Kamolke University, Leuven, Belgium) was used in all the experiments and these were carried out between passages 127–134. Cells were routinely maintained in a 25-cm² tissue culture flask (Falcon, BD Biosciences, Oxford, UK) at 28°C in a humidified incubator with 1% CO₂ in air as described previously (11, 41). The culture medium was a mixture of 1 part Ham’s F-12 medium with L-glutamine (GIBCO, Parsley, UK) and 1 part L-15 medium with L-glutamine (GIBCO) modified for amphibian cells at pH 7.4. In addition to these components, the culture medium was also supplemented with 10% fetal bovine serum (GIBCO), 200 mg/ml streptomycin, and 200 U/ml penicillin (GIBCO). The final growth medium osmolality was measured using an automatic Micro-Osmometer (type 13/13DR-Autocal, CAMLAB, Cambridge, UK) and found to be 260 mosmol/kgH₂O. When the cells were 90–95% confluent, they were passaged or seeded on the membrane filter inserts (23-mm diameter, 0.4-µm pore, 1.6 × 10⁶ pores/cm², Falcon, BD Biosciences) at a density of 2 × 10⁵ cells/cm² for 10–12 days until formation of a tight epithelium that exhibited a stable transepithelial electrical resistance. Well-developed A6 cell monolayers were then pretreated with aldosterone using a modified protocol reported by Kemendy et al. (18). In brief, this involved the tight A6 cell monolayer being maintained for 24 h in the growth medium together with 10% fetal calf serum as above, but supplemented with 1.5 µM aldosterone (Sigma, Dorset, UK) added to the basolateral compartment of the cell monolayer. After 24 h, the cell monolayer was incubated in a serum-free medium, but still in the presence of 1.5 µM aldosterone for a further 24 h to facilitate the maximal hormonal responsiveness. The cells were then grown in a culture medium which was free of both serum and aldosterone and incubated for 48 h. Before being used in the experiments, A6 cells were bathed with a modified isosmotic L-15 medium (GIBCO) with a final osmolality of ~260 mosmol/kgH₂O that was verified with the automatic Micro-Osmometer (CAMLAB).

SICM. We obtained topographic images of A6 cells using an SICM (Ionoscope, London, UK) as described previously (11, 20, 41). Briefly, the SICM uses a nanopipette, mounted on a three-axis piezo translation stage and arranged perpendicularly to the cell monolayer as a scanning probe. The SICM feedback control system keeps the ion current through the pipette constant to approach and scan over cells while maintaining a constant separation distance, within approximately the nanopipette tip internal radius from the A6 cell surface. The SICM then produces a three-dimensional topographical image of the A6 cell membrane surface.

The SICM nanopipettes were pulled from borosilicate glass capillaries (Intrafil, 1.0-mm OD × 0.58-mm ID, Intracel, Hertz, UK), using a laser-based electrode puller (P-2000, Sutter Instrument, Novato, CA). To perform the scanning, we used a pipette with an ~50-nm tip internal radius and electrical resistance of ~100 MΩ when submerged in the L-15 medium.

Measurement of transepithelial electrical resistance and calculation of transepithelial electrical conductance. A well-developed A6 epithelial cell monolayer has been demonstrated to possess a stable and high transepithelial electrical resistance (Rₑ) when cultured on membrane filters (12). In our study, the Rₑ was measured using a commercial Endohm 24 chamber connected to an Epithelial Volt Ohmmeter (EVOM, World Precision Instruments, Hertfordshire, UK) as described previously (11). Briefly, membrane filter inserts with cultured A6 cells were transferred to the Endohm 24 chamber for measurement. The Rₑ measurements were performed in L-15 medium at room temperature after the EVOM reading became stable (~30–60 min). The measured resistance output from the EVOM was sampled at 1 Hz with a DIGidata 1322A 16-bit data-acquisition system and pCLAMP 9.2 software (Axon Instruments, Union City, CA). Rₑ was expressed in 1/µΩ·cm² and determined by multiplying the measured resistance by the effective membrane area of the A6 cell monolayer, which was calculated to be 4.19 cm² of the cell culture membrane filter supports. It was essential that the cell monolayer was relatively impermeable during all transepithelial Na⁺ transport and P2X₄ receptor assessment experiments. Only cells with a stable initial Rₑ of >8,000 1/µΩ·cm² were chosen for these experiments. Transepithelial electrical conductance (Gₑ) was then calculated using Gₑ = 1/Rₑ, and was expressed in Siemens per square centimeter (S/cm²).

Indirect pharmacological screening for purinergic receptors involved: P2X₄ receptor agonists and antagonists. In this study, we were only concerned with the effect of extracellular ATP in the basolateral compartment. Since the inhibitory effect of extracellular ATP on the epithelial apical membrane has been previously demonstrated (9, 22, 35, 39), to avoid any diffusion of ATP agonists from the basolateral side to the apical side, these experiments were performed under continuous perfusion of the apical bath solution (2 ml) with L-15 medium at a rate of 5 ml/min by gravity feed from a connected reservoir.

The agonists used to screen for P2X₄ receptors were ATP analogs including ATPγS, 2-methylthio-ATP (2MeSATP), α,β-methylene-ATP (αβmeATP), and CTP (3, 10, 27, 36). Because β,γ-methylene-ATP (βγmeATP) is another stable analog of ATP, which is more potent at P2X receptors than P2Y receptors (29), βγmeATP was also used. All agonists were purchased from Sigma and used at a final concentration of 50 µM. Ivermectin (10 µM, Sigma), which has been proven to potentiate the activity of P2X₄ receptors (26–28), was also used in our study.

P2X₄ receptors also have their own characteristic profile with respect to the effects of different ions. Zn²⁺ potentiates the cation conductance induced by ATP at cloned P2X₂ and P2X₃ receptors, but Cu²⁺ has been reported to increase the ATP-activated current through P2X₄ receptors but had no effect at P2X₄ receptors (27). This ion dependence allows P2X₄ receptors to be distinguished from other P2X receptors. In this study, either 10 µM ZnCl₂ or 50 µM CuCl₂ (27) was added to the basolateral side of the A6 monolayer during 2MeSATP stimulation.

An alternative way to get information about the activation of purinergic receptors is to use antagonists that block P2 receptors. As selective P2X₄ receptor antagonists are not readily available, we used the generic P2 receptor blocker suramin (100–300 µM) (14) used either alone or together with 100–300 µM PPADS (nonspecific P2 receptor antagonist) (32, 40) and added to the apical side of the A6 cell monolayer. Since the inhibitory effect of extracellular ATP on the epithelial basolateral membrane has been previously demonstrated (9, 22, 35, 39), to avoid any diffusion of ATP agonists from the basolateral side to the apical side, these experiments were performed under continuous perfusion of the apical bath solution (2 ml) with L-15 medium at a rate of 5 ml/min by gravity feed from a connected reservoir.

Effect of amiloride. To determine whether any observed effect of ATP analogs involved an action via ENaCs, experiments were repeated with the addition of 10 µM amiloride (Sigma) to the apical compartment of the A6 cell monolayer. During the experiments, amiloride washout was accomplished by continually perfusing the apical side (2 ml) of the A6 cell monolayer with L-15 medium at a rate of 6 ml/min by gravity feed from reservoirs connected to the Endohm 24 chamber (World Precision Instruments), combined with a suction pump system to remove the solution and maintain the level of the bath stable. Complete removal of amiloride blockade took about 2–3 min.

Data analysis. All data were expressed as the average of n experiments (means ± SE). For comparison of the data to assess the statistical significance, Student’s t-test was used. The P value and the number of experiments or the numbers of measurements contributing to the mean values reported are given in the tables as appropriate. A difference between means at the level of P < 0.05 was considered significant; P < 0.01 was highly significant; and P < 0.001 was very highly significant. The statistical analyses were performed using Microcal Origin, version 5.0 (Microcal Software, Northampton, MA).
RESULTS

We have recently found that the basolateral addition of ATPγS (50 μM) induced progressive lateral cell contraction accompanied by apical membrane expansion in an A6 monolayer (11). To further investigate the P2X receptors involved in our findings, we used SICM to observe the changes in membrane structures of A6 cells in response to another nonhydrolyzable form of ATP, 2MeSATP, which has been demonstrated to be the most potent agonist of recombinant and native P2X4 receptors (29, 34). Figure 1A presents six selected time-sequence SICM images of the same area of an A6 cell monolayer taken over a 2-h period. The first two control frames (0:00–0:18) show a flat and uniform cell monolayer with well-developed tight junctions between cells and pronounced microvilli covering the cell membrane. After the basolateral addition of 2MeSATP (50 μM), the cell morphology changed significantly, with progressive lateral cell contraction and apical membrane elevation (frame 0:36 to 1:12). The last frame (1:48) shows gradual recovery of the original morphology of the cells. To highlight the typical changes in cell apical membrane structures, topographical profile changes in a selected A6 cell marked by an asterisk in Fig. 1A (frame 0:00) are presented (Fig. 1B). The scanned profiles are across two points of tight junction joints a and b, each sited on the points of apposition of the asterisk-marked cell and two other neighboring cells, represented by the dashed line marked on frames 0:18 and 1:12. Compared with the profile of the control frame (frame 0:18; Fig. 1B, top trace), the profile line scanned after 2MeSATP stimulation (frame 1:12; Fig. 1B, bottom trace) shows clear lateral contraction (note the arrow-marked position change in tight junction joint b) accompanied by vertical apical membrane elevation. Such 2MeSATP-induced cell morphological changes are very similar to those membrane structure changes that we have previously linked with ENaC activity of A6 cells in response to aldosterone, hypotonic stress, or ATPγS (11).

We further observed that the addition of 50 μM 2MeSATP to the basolateral side of the A6 cells, as with our published results of experiments in response to ATPγS (11), produced a rapid rise in cell monolayer Gt that was inhibited by the ENaC inhibitor amiloride (Fig. 2).

The P2X receptor family have a diverse pharmacology, and the agonist potency orders vary significantly between P2X subtypes (26). The agonist potency profile of the cloned P2X4 receptors is ATP ≥ 2MeSATP > CTP > α3meATP (10, 27, 36). ATPγS is also an agonist at recombinant P2X4 receptors, with a potency generally less than that of ATP (3). To test whether the P2X4 receptor is specifically involved in sodium transport, we tested its agonist sensitivity by measuring the

Fig. 2. Effects of basolateral 2MeSATP on the transepithelial electrical conductance (Gt) of A6 cell monolayers. Typical time-dependent effect of the basolateral addition of 50 μM 2MeSATP on Gt increases in the A6 monolayer. The major part of increases in Gt was amiloride sensitive. 2MeSATP was applied for the duration, as indicated by the solid line above the trace. The thick horizontal lines on the bottom of figure indicate the application of amiloride to the apical side of the A6 cell monolayer.
amiloride-sensitive sodium conductance changes ($\Delta G_i^{ENaC}$) of the A6 monolayer. The basolateral addition of selected P2X agonists (50 μM), including 2MeSATP, ATPyS, βymeATP, αβmeATP, and CTP, all significantly increased the $\Delta G_i^{ENaC}$ of the cell monolayer (Table 1). The sensitivity of the involved P2X receptors to 2MeSATP, ATPyS, and CTP in the A6 monolayer (2MeSATP $\geq$ ATPyS $>$ CTP) displays a similar agonist potency profile to that of cloned P2X4 receptors. However, compared with the cloned P2X4 receptors, the A6 cell monolayer had a notably higher sensitivity for the agonist βymeATP and αβmeATP (2MeSATP $>$ βymeATP $\geq$ αβmeATP $>$ CTP).

P2X4 receptors also have their own characteristic profile with respect to the effects of different ions. Zn$^{2+}$ potentiates the cation conductance induced by ATP at cloned P2X2 and P2X3 receptors, and Cu$^{2+}$ has been reported to increase the ATP-activated current through P2X2 receptors but had no effect at P2X4 receptors (10, 27, 36). This ion dependence allows P2X4 receptors to be distinguished from other P2X receptors. In our study, zinc (Fig. 3A) potentiated the 2MeSATP-induced amiloride-sensitive sodium transport of the A6 monolayer but not Cu$^{2+}$ (Fig. 3B). P2X4 receptors have been demonstrated to be insensitive to the known P2 receptor antagonists suramin and PPADS (5, 27). Figure 3C indicates that the 2MeSATP-evoked $G_i$ changes in the A6 monolayer cannot be inhibited with suramin and PPADS (100–300 μM). Moreover, the effects of suramin and PPADS on the 2MeSATP-evoked sodium transport of the A6 monolayer was similar to that found by Bo et al. (3) with cloned P2X4 receptors, namely, that both antagonists did not reduce but further increased ATP-mediated effects.

Ivermectin is widely used in human and veterinary medicine as an antiparasitic agent and has been proven to potentiate the activity of P2X4 receptors in homomeric and heteromeric configuration, but does not affect extracellular ATP-induced currents in cells expressing other types of purinergic receptors (26–28). Compared with the 50 μM 2MeSATP treatment alone, preincubating the basolateral side of A6 monolayers with 10 μM ivermectin for 5 min had a significant potentiating action on 2MeSATP-induced $\Delta G_i^{ENaC}$ of the A6 monolayer (Table 2). A semiselective P2X4 antagonist and specific P2X7 antagonist, BBG (15), was used to further assess the P2X subtypes involved in the 2MeSATP-induced $G_i^{ENaC}$ changes in the A6 monolayer (Table 2). Similar to the results published by Jiang et al. (15) using rat and human P2X4 receptors, preincubating the A6 monolayer with 10 μM BBG did not totally inhibit the 2MeSATP-induced amiloride-sensitive $G_i$ increase but significantly reduced the effect of 2MeSATP on $\Delta G_i^{ENaC}$.

### DISCUSSION

Our previous work has suggested that aldosterone acts via an autocrine/paracrine purinergic system to stimulate sodium transport across renal epithelia (11). In these studies, the release of ATP into the basolateral compartment led to the contraction of the cell, with elevation of the apical membrane and associated opening of the ENaC. We have now carried out further studies which suggest that the purinergic receptor involved is P2X4 like. The P2X4 receptor appears to be the most widespread member of the P2X ligand-gated ion channel superfamily (2, 3, 26). Activation of P2X4 receptors leads to the opening of nonselective cation channels; the recombiant P2X4 receptor is most potently activated by 2MeSATP (3, 33).

In our study, 2MeSATP addition to the basolateral side of the A6 cell monolayer produced progressive cell contraction and apical membrane protrusion. Similar morphological changes, induced with ATPyS and other physiological stimuli, have been shown in our previous experiments to be clearly linked with ENaC activity in A6 cells (11). The agonist order of potency determined by amiloride-sensitive $G_i$ changes in A6 monolayers to 2MeSATP, ATPyS, and CTP (2MeSATP $\geq$ ATPyS $>$ CTP) displays a similar agonist potency profile to that of cloned P2X4 receptors. However, we found a notably higher sensitivity for the agonists βymeATP and αβmeATP.

The marked response of the A6 cells to βymeATP and αβmeATP, which should be inactive or weak agonists at P2X4 receptors (3, 26, 29), raises the question as to whether the effect is via any of the currently known P2X receptors. The subtypes that are sensitive to αβmeATP, such as P2X1 or P2X3 receptors, are not likely to be involved, because of their high sensitivity to suramin and PPADS. However, it is known that the ability of added ATP agonists to mimic intercellular signaling molecules also depends on the rates of ATP release, hydrolysis by ecto-ATPases, and the rate of ATP synthesis by ecto-nucleotide diphosphokinases (17). Methylene ATP analogs, such as βymeATP and αβmeATP, as their names suggest, possess a methylene group substituted for the oxygen in the phosphodiester bridge between the phosphate moieties of ATP. Since this renders such analogs relatively resistant to hydrolytic attack by nucleotide phosphohydrolases, they have been used as stable agonists for certain P2 receptors and also as more effective inhibitors of ATP degradation mediated by ecto-ATPases (17). When 50 μM βymeATP and αβmeATP were utilized as P2X agonists, βymeATP more strongly inhibits ecto-ATPase than αβmeATP in some cell types (17). Moreover, both methylene ATP analogs may also secondarily induce extracellular ATP synthesis and accumulation of ATP, the most potent agonist for P2X4 receptors (17). It is also clear

### Table 1. Effects of various P2X agonists (50 μM) on the amiloride-sensitive $G_i$ changes in A6 monolayers

<table>
<thead>
<tr>
<th>Group</th>
<th>Status</th>
<th>No. of Experiments</th>
<th>Normalized $\Delta G_i^{ENaC}$</th>
<th>P Value vs. Control</th>
<th>P Value vs. 2MeSATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>9</td>
<td>1.049±0.034</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>2MeSATP</td>
<td>9</td>
<td>2.803±0.277</td>
<td>P &lt; 0.001</td>
<td>Not significant</td>
</tr>
<tr>
<td>3</td>
<td>ATPyS</td>
<td>9</td>
<td>2.738±0.364</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>4</td>
<td>βymeATP</td>
<td>8</td>
<td>2.403±0.291</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>5</td>
<td>αβmeATP</td>
<td>7</td>
<td>2.071±0.125</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>6</td>
<td>CTP</td>
<td>7</td>
<td>1.499±0.146</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE. 2MeSATP, 2-methyis-ATP; $\Delta G_i^{ENaC}$, amiloride-sensitive transepithelial conductance ($G_i$) changes.
that βymeATP can act directly as an agonist at adenosine/P1 receptors (29). These receptors have also been demonstrated to be involved in the purinergic regulation of Na⁺ transport in A6 cells (11, 23).

Several further distinguishing features of ATP-evoked sodium transport in A6 cells allow us to conclude that it results from the activation of P2X₄-like receptors. First is the potentiation by ivermectin and the lack of any blockade by suramin or PPADS (5, 26). Preincubating the basolateral side of the A6 monolayers with 10 μM ivermectin had a significant potentiating effect on the 2MeSATP-induced amiloride-sensitive Gₛ of the A6 monolayer. 2MeSATP responses, again indicating that P2X₄ receptors are actually potentiated by suramin and PPADS in A6 cells. P2X₄ is relatively insensitive to the conventional antagonists suramin and PPADS, possibly because of the absence of a lysine residue in the receptor (5). Moreover, it has been suggested that the insensitivity of purinergic receptors to suramin is due to its inhibition of the ecto-ATPases, which break down ATP (8).

In our study, Cu²⁺ had no effect and Zn²⁺ potentiated the 2MeSATP responses, again indicating that P2X₄ receptors are involved. Moreover, preincubating the A6 monolayer with a semiselective P2X₄ antagonist and specific P2X₇ antagonist, BBG, significantly reduced but did not totally inhibit the 2MeSATP-induced sodium conductance increase, which also suggests the involvement of P2X₄-like receptors.

Studies of the functional properties of heterologously expressed P2X receptors, together with examination of their distribution in native tissues, suggests P2X receptors are likely to occur as both homo- and heteromultimers in vivo (24, 26). There is some evidence that P2X₄ may heteropolymerize with P2X₆ receptors since they are often found together in native tissues, and can be coimmunoprecipitated (27). Functionally expressed homomeric P2X₆ receptors are sensitive to PPADS and relatively insensitive to suramin and may contribute βmeATP sensitivity to heteromeric P2X₄₆ receptors (16, 19). However, those heteromeric P2X₄₆ receptors have been demonstrated to have a higher sensitivity to suramin and PPADS (21), so these homomeric P2X₆ and heteromeric P2X₄₆ receptors are not likely to be involved because of the insensitivity of the A6 cells to suramin and PPADS in our study. However, P2X₄₆ heteromeric receptors can also be formed (37), and it is even possible that all three subtypes, P2X₄₆, might coassemble to form a novel phenotype (35). We cannot rule out the possibility that the widespread P2X₄ subunit serves as the principal subunit to form possibly novel heteromers with other P2 and P1 subunits in the distal nephron.

Unwin and Burnstock’s group (38) found that only P2X₄ and P2X₆ receptors were present on the basolateral membrane of the rat distal tubular epithelium. The fact that our results indicate that there are P2X₄-like receptors in the A6 epithelial cell line derived from frog distal tubule is consistent with their findings. Given our results indicating that ATP release from renal epithelial cells plays a critically important role in the mechanism of action of aldosterone (11), the precise location of these purinergic receptors is of interest because of their likely role in regulating salt and water balance. We suggest that sodium reabsorption is stimulated by activation of the P2X₄ receptors along the tubular basolateral side of the distal nephron, making manipulation of these receptors of interest in

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**Fig. 3. Effects of Zn²⁺, Cu²⁺, suramin, and PPADS on the 2MeSATP-evoked amiloride-sensitive Gₛ in an A6 monolayer.**

A: adding 10 μM ZnCl₂ to the basolateral side potentiated the 2MeSATP-induced amiloride-sensitive Gₛ increase. B: adding 50 μM CuCl₂ to the basolateral side had no effect on the 2MeSATP-induced amiloride-sensitive Gₛ increase. C: 100 μM suramin and PPADS not only did not reduce but further increased amiloride-sensitive Gₛ of the A6 monolayer. 2MeSATP, Zn²⁺, Cu²⁺, suramin, and PPADS were applied for the duration, as indicated by the marked solid line above the trace. The thick horizontal lines on the bottom of figure indicate the application of amiloride to the apical side of the A6 cell monolayer.

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**Table 2. Ivermectin (10 μM) potentiated and BBG (10 μM) reduced the 2MeSATP-induced amiloride-sensitive Gₛ changes in A6 monolayers**

<table>
<thead>
<tr>
<th>Group</th>
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<th>No. of Experiments</th>
<th>Normalized ΔGₛ</th>
<th>P Value vs. 2MeSATP</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2MeSATP</td>
<td>9</td>
<td>2.803±0.277</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>Ivermectin + 2MeSATP</td>
<td>8</td>
<td>3.675±0.295</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>BBG + 2MeSATP</td>
<td>9</td>
<td>1.581±0.181</td>
<td>&lt;0.001</td>
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</table>

Values are means ± SE. BBG, brilliant blue G.
conditions with aldosterone excess or enhanced renal responsiveness to mineralocorticoids. Better agonists and antagonists are necessary to investigate the physiology of native P2X4 receptors, and results with these may point to new means of treatment.

Previous studies have examined the effect of nucleotides on Na+ transport in collecting duct cells when added to the apical side of the cell (9, 22, 25, 35, 39). In contrast to our results, these show that ATP inhibits rather than stimulates Na+ reabsorption. The P2Y2 receptor is the prominent luminal receptor in many epithelia (22), and ATP-induced inhibition of Na+ reabsorption in the distal nephron is normally thought to be via P2Y receptors. This might suggest that epithelial cells had a reciprocal mechanism controlling Na+ in which luminal ATP, acting via P2Y receptors, inhibited transport while basolaterally released ATP stimulated it via P2X4 receptors. However, there is clear evidence that P2X receptors are also present on the apical membrane (25, 35). These have been functionally localized using selective P2 agonists (25). They suggested that P2X as well as P2Y receptors in the apical membrane are involved in the inhibition of Na+ transport and Cl− stimulation. To confirm the presence of the purinergic receptors in their mouse inner medullary collecting duct cell line, McCoy and colleagues (25) used RT-PCR amplification and were able to identify the P2X and P2Y receptor mRNA subtype; both P2X (P2X3 and P2X4) and P2Y (P2Y1 and P2Y2) receptor mRNAs were expressed. These experiments help to confirm the presence of the receptor subtypes but do not identify their cellular or subcellular location.

At first sight, it might seem unlikely that P2X receptors might play an inhibitory role when located apically and a stimulatory one when in the basolateral cell membrane. Wildman and colleagues (39) have provided a potential explanation for this. They looked at the regulatory interdependence of cloned epithelial Na+ channels and P2X receptors using coexpression in X. laevis oocytes. The experiments showed that P2X2, P2X2/6, P2X4, and P2X4/6 receptors when activated inhibited amiloride-sensitive Na+ transport. The P2X-mediated inhibition was found to be due to removal of ENaC from the plasma membrane. This removal occurred at a much faster rate than that via the influx of Na+ through ENaC itself. They suggested that P2X receptor ion channels involved in Na+ transport might be “in close proximity to ENaC in such a way that activation of the P2X receptors results in a localized delivery of sodium ions to a site on or near ENaC that is particularly sensitive to increases in [Na+]l” (39).

This suggests that the physical relationship of the plasma membrane P2X receptors to ENaC might determine their effect on Na+ transport. Those receptors in the apical membrane exposed to luminal ATP might inhibit reabsorption by removal of ENaC from the cell surface. By contrast, basolateral P2X receptors when activated can stimulate Na+ reabsorption via the aldosterone mechanism that we have described. Further work needs to be done to determine whether this elegant reciprocal sodium control hypothesis, potentially using the same purinergic receptor for a different purpose, is correct.

In summary, we have shown the existence of P2X4-like receptors in the A6 cell basolateral membrane. It has been demonstrated that the basolateral P2 receptors facilitate Na+ transport, and there is well-documented evidence for apical P2 receptor-mediated inhibition of Na+ transport. The complexities of several ATP release mechanisms and multiple types of P2 receptors expressed by a given renal epithelial cell along the nephron have also been delineated (1, 4, 6, 13, 31, 38). This suggests an elegant reciprocal purinergic system acting both at a basolateral and apical location for control of Na+ transport. Such a system would require a mechanism within the cell that resulted in either basolateral or apical release of ATP for the autocrine and/or paracrine regulation of renal tubular function.

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REFERENCES


