Sodium-Dependent Regulation of Renal Amiloride-Sensitive Currents by Apical P2 Receptors

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ABSTRACT

The epithelial sodium channel (ENaC) plays a major role in the regulation of sodium balance and BP by controlling Na⁺ reabsorption along the renal distal tubule and collecting duct (CD). ENaC activity is affected by extracellular nucleotides acting on P2 receptors (P2R); however, there remain uncertainties over the P2R subtype(s) involved, the molecular mechanism(s) responsible, and their physiologic role. This study investigated the relationship between apical P2R and ENaC activity by assessing the effects of P2R agonists on amiloride-sensitive current in the rat CD. Using whole-cell patch clamp of principal cells of split-open CD from Na⁺-restricted rats, in combination with immunohistochemistry and real-time PCR, we found that activation of metabotropic P2R (most likely the P2Y2 and/or 4 subtype), via phospholipase C, inhibited ENaC activity. In addition, activation of ionotropic P2R (most likely the P2X4 and/or 4/6 subtype), via phosphatidylinositol-3 kinase, either inhibited or potentiated ENaC activity, depending on the extracellular Na⁺ concentration; therefore, it is proposed that P2X4 and/or 4/6 receptors might function as apical Na⁺ sensors responsible for local regulation of ENaC activity in the CD and could thereby help to regulate Na⁺ balance and systemic BP.


ATP and other nucleotides are released from epithelial cells to activate, in an autocrine and/or paracrine manner, a variety of surface membrane receptors known as P2 receptors (P2R). According to their sequence identity and signal transduction, P2R are subdivided into two types: Ionotropic P2X (P2XR) and metabotropic P2Y (P2YR). There are seven P2XR subunits that form seven homomeric (P2X1 to 7), seven established heteromeric (P2X1/2, 1/4, 1/5, 2/3, 2/6, 4/6, and 4/7), and an additional six predicted heteromeric (P2X1/3, 1/6, 2/5, 3/5, 4/5, and 4/6) nonselective cation channel assemblies. Characterized P2XR have distinct biophysical and/or pharmacologic profiles. In addition, there are eight G protein–coupled P2YR (P2Y1, 2, 4, 6, 11, 12, 13, and 14), also with distinct pharmacologic profiles; they couple to either phospholipase C (PLC) to increase inositol triphosphate (IP₃) levels or adenyl cyclase to change cAMP levels. Activation of P2R can alter the activity of certain epithelial cell membrane transport proteins. A key transport protein shown to be affected by the activation of P2R is the amiloride-sensitive epithelial Na⁺ channel (ENaC). ENaC is expressed at the apical membrane of principal cells (PC) in the collecting duct (CD), and its activity is an important factor in the regulation of arterial BP, because it...
determines the extent of Na\(^+\) reabsorption from tubular fluid along the distal nephron.\(^9,10\)

Given the importance of ENaC in controlling arterial BP, it is perhaps not surprising that there have been several studies on the modulation of its activity by P2R in the CD. To date, P2R-mediated modulation of ENaC activity has been demonstrated by means of electrophysiologic techniques in murine and amphibian immortalized renal CD/distal nephron cell lines\(^7,8,11\textendash}15\) and in the \textit{Xenopus} oocyte heterologous expression system (using recombinant rat P2XR and ENaC proteins).\(^6\) It has also been reported in the CD of rat\(^17\) and mouse\(^18,19\) using \textit{in vivo} and \textit{in vitro} luminal perfusion studies, respectively. The prevailing view from these studies is that P2R inhibit ENaC activity; however, open issues remain with regard to the P2R subtype(s) involved, the membrane localization of P2R in the CD, and the intracellular mechanism(s) responsible for inhibition.

To address these issues, we investigated the relationship between the activation of apical P2R and ENaC activity in PC (\textit{in situ}) in microdissected split-open rat CD, using whole-cell patch-clamp electrophysiology, in combination with real-time PCR and immunohistochemical techniques. Understanding the possible regulation of ENaC activity by P2R will extend our insight into the mechanism(s) responsible for inhibition of ENaC activity; however, open issues remain with regard to the P2R subtype(s) involved, the membrane localization of P2R in the CD, and the intracellular mechanism(s) responsible for inhibition.

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RESULTS

Urinary Na\(^+\) Levels

As anticipated, urinary Na\(^+\) concentrations in Na\(^+\)-restricted rats were significantly less at day 10 (4.3 ± 0.9 mmol/L; \(n = 5\)) than those in Na\(^+\)-replete rats (115 ± 11 mmol/L; \(n = 5\); \(P < 0.01\)). This degree of Na\(^+\) restriction is known to enhance the expression of functional ENaC in the apical membrane of PC in the rat CD.\(^20\textendash}22\)

**Immunohistochemical Co-localization of P2R and ENaC Subunits**

Positive immunofluorescence (localized to membrane domains and/or intracellular regions of PC and/or intercalated cells [IC]) was seen for P2X\(_2,3,4,5\), and \(\gamma\) ion channel subunits and P2Y\(_{2,4,6,9,11,12,13}\) metabotropic receptors in the CD of Na\(^+\)-replete rats (identified by positive aquaporin 2 [AQP2] staining). The same P2R subtypes were localized to the CD of Na\(^+\)-restricted rats but with the addition of P2X\(_4\) and loss of P2X\(_3\) ion channel subunits (Table 1). For all P2R antibodies used, preincubation with their respective antigenic peptides abolished positive immunofluorescence (see Supplemental Data 1). Positive immunofluorescence was also seen for \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC subunits in the CD of both Na\(^+\)-replete and Na\(^+\)-restricted rats; this immunofluorescence appeared stronger in the PC of Na\(^+\)-restricted rats.

With respect to PC only, in Na\(^+\)-replete rats strong immunostaining for P2X\(_4\) and \(\gamma\) and for P2Y\(_{4,6,9}\) and \(\alpha\) was consistently observed in the apical domain of PC throughout the CD, and weaker apical staining was also seen for P2X\(_2\) and P2Y\(_2\) (both localized to the medullary CD only) and \(\alpha\)-ENaC. Strong basolateral immunostaining for P2X\(_4\) and \(\gamma\) was seen in all PC; basolateral staining for P2Y\(_2\) was evident only in medullary CD PC. Punctate intracellular staining (\textit{i.e.}, staining that could not be confidently localized to the plasma membrane of PC) was seen for P2X\(_2\) and \(\beta\); P2Y\(_{2,4,6,9,12,13}\) (Table 1); and \(\beta\)- and \(\gamma\)-ENaC.

In PC from Na\(^+\)-restricted rats, consistently positive

| Table 1. Comparison of immunohistochemical localization of P2R in the CD of rats maintained on a normal (0.5% NaCl) or low (0.01% NaCl) Na\(^+\) diet for 8 to 10 d* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **P2R** | **CCD** | **OMCD** | **IMCD** | **CCD** | **OMCD** | **IMCD** |
| P2X\(_1\) | – | – | – | IC | IC | a/IC |
| P2X\(_2\) | int | int | int/a | int | int | int/a |
| P2X\(_3\) | – | – | – | – | – | – |
| P2X\(_4\) | A/B | A/B | A/B | A/B | A/B | A/B |
| P2X\(_5\) | int | int | – | – | – | – |
| P2X\(_6\) | A/B | A/B | A/B | A/B | A/B | A/B |
| P2X\(_7\) | – | – | – | – | – | – |
| P2Y\(_1\) | – | – | – | – | – | – |
| P2Y\(_2\) | int | int/a/b | A/B | int | int/a/b | a/B |
| P2Y\(_4\) | A | A | A | A | A | A |
| P2Y\(_5\) | A/int | A/int | A/int | int | int | int |
| P2Y\(_7\) | – | – | – | – | – | – |
| P2Y\(_8\) | – | – | – | – | – | – |

* AQP2 was used as a marker of CD. Kidneys were perfusion-fixed with paraformaldehyde (4%) and sliced to 8 \(\mu\)m thickness. Only cortical collecting duct (CCD) and outer medullary collecting duct (OMCD) tubules were used in patch-clamp and real-time PCR investigations. –, immunofluorescence (IF) not detected; A, positive IF (+IF) localized to apical membrane; a, weak +IF localized to apical membrane; B, +IF localized to basolateral membrane; b, weak +IF localized to basolateral membrane; int, +IF localized to intracellular loci; IC, +IF localized to intercalated cells and not PC; IMCD, inner medullary collecting duct.
β-ENaC and γ-ENaC staining became evident in the apical domain throughout the CD, and intracellular immunostaining for P2X5 disappeared. Localization and staining of all other P2X subunits was unchanged, except for the appearance of weak expression of P2X6 subunits in the inner medullary CD (Figure 1A and B). With respect to P2YR, P2Y3, 4, 11, 12, and 13 staining was unchanged (Figure 1C) and P2Y6 showed restricted intracellular staining (see Table 1). As an incidental finding, positive P2X1 staining became evident in IC, but this was not investigated further.

Expression of P2R mRNA in CD
To quantify the relative abundance of P2R mRNA in microdissected CD (i.e., PC and IC) of Na+-replete and Na+-restricted rats, we calculated a ratio of the P2R gene of interest to a constitutively expressed housekeeping gene (hypoxanthine phosphoribosyltransferase [HPRT]). No suitable primers were available for P2X1, 3, 5, and 7 and P2Y1 mRNA levels for P2Y4 receptors were significantly increased in Na+-replete rats (Figure 2) corresponding to broadly agrees with the immunohistochemical findings. In contrast, significant amounts of mRNA (all of a similar value [i.e., approximately 2 arbitrary units]; for calculation of number of transcripts per cell, see Supplemental Data 2) were detected for P2X4 and P2Y2 and 6 in CD of Na+-replete rats (Figure 2). CD from Na+-restricted rats showed a significant increase in abundance of P2X1 mRNA (by two-fold; n = 6; P < 0.01) but no change in P2Y2 or P2Y6 mRNA levels. Whereas P2X1 and 6 and P2Y4 mRNA levels were deemed insignificant in Na+-replete rats, Na+-restricted rats showed marked abundance of mRNA for each of these subtypes (Figure 2).

Whole-Cell P2R-Mediated Currents
Whole-cell perforated patch-clamp recording was possible from individual PC in the rat CD because electrical coupling is very low between these cells, as reported by Frindt et al. As also reported by Frindt et al., the whole-cell capacitance, an electrical index of membrane area, was found to be significantly greater in PC from Na+-restricted rats (32 ± 6 pf; n = 40) than from Na+-replete rats (15 ± 4 pf; n = 40). A low-Na+ diet approximately doubles the combined surface area of a PC’s apical and basolateral membranes.

Figure 3 shows ATP- and other P2R agonist-evoked (all at 10 μM) whole-cell inward currents in voltage-clamped (holding potential [Vh] = −60 mV) PC in situ from rats maintained on a Na+-replete diet. Agonists were selected according to Table 2.

In PC from Na+-replete rats, the amplitudes of 10 μM agonist-evoked currents were 581 ± 19 pA (for ATP; n = 10), 468 ± 65 pA (ATPγS; n = 5), 232 ± 45 pA (Ap6A; n = 5), 600 ± 123 pA (UTP; n = 8), 594 ± 21 pA (2meSADP; n = 5), 28 ± 11 (2meSATP; n = 5), and 114 ± 39 pA (BzATP; n = 3; Figure 4A, data normalized for cell size). Currents evoked by the application of Ap6A, UTP, BzATP, and 2meSADP were completely abolished after preincubation (3 min) and co-application of DIDS (4,4’-Di-isothiocyanatostilbene-2,2’-disulfonic acid; a Ca2+-activated Cl− channel blocker; 100 μM; n = 3 in each case; example in Figure 3B), suggesting that their action was mediated through G protein–coupled P2YR and the involvement of Ca2+-dependent chloride “reporter” currents. Currents evoked by 2meSATP were unaffected after DIDS treatment (Figure 3B).

In PC of Na+-restricted rats, the amplitudes of 10 μM agonist-evoked currents were 386 ± 20 pA (ATP; n = 10), 380 ± 25 pA (ATPγS; n = 5), 273 ± 48 pA (UTP; n = 6), 388 ± 21 pA (2meSATP; n = 5), and

**Figure 1.** Confocal images demonstrating positive immunofluorescence staining for P2R in the cortical CD of Na+-replete rats. (A) Apical and basolateral staining (FITC) for P2X5 subunits in PC, similar to that seen for P2X6 (B). (C) Apical staining (FITC) for P2Y4 receptors in PC. In all experiments, CD were identified by positive AQP2 co-staining. L, lumen.

**Figure 2.** Comparison of P2R mRNA levels in microdissected CD from Na+-replete and Na+-restricted rats. Data presented as a ratio between the P2R gene of interest and the constitutively expressed housekeeping gene HPRT. (A) mRNA levels for P2X1, 4, and 6 subunits were significantly increased in Na+-restricted rats (n = 6; P < 0.01). (B) mRNA levels for P2Y4 receptors were significantly increased in Na+-restricted rats (n = 6; P < 0.01).
In Na⁺-restricted rats, C/R curves were shifted approximately 0.5 log units to the left for all full agonists, with the exception of BzATP, for which there was no significant change (Figure 5B). The other difference was that Ap₆A activity was virtually abolished. EC₅₀ values were as follows: ATP 0.3 ± 0.5 μM (n = 5); ATPγS 0.4 ± 0.3 μM (n = 5); UTP 0.9 ± 0.4 μM (n = 5); 2meSATP 0.4 ± 0.4 μM (n = 5); and BzATP 8 ± 2 μM (n = 3).

**Current/Voltage Relationships**

Steady-state current/voltage (I/V) relationships for whole-cell currents from PC were investigated for amiloride-sensitive currents (ENaC-mediated currents [Iₐm-s]). Figure 6 shows typical amiloride-sensitive I/V relationships recorded from Na⁺-replete and Na⁺-restricted rats. Data for I/V curves were calculated as the difference between total current amplitude and current amplitude in the presence of 10 μM amiloride (blocker of ENaC activity; as shown in Figure 6A from a Na⁺-restricted rat). I/V curves for Iₐm-s showed inward rectification, with mean reversal potentials (Eᵣₑᵥₑ) of −4 ± 4 mV (n = 6) and 58 ± 7 mV (n = 46) in PC from Na⁺-replete and Na⁺-restricted rats, respectively (Figure 6B). In Na⁺-replete rats, Iₐm-s was close to zero (n = 6), indicating a paucity of apical ENaC. The effect of amiloride (in Na⁺-restricted rats) was found to be reversible, but, with time, Iₐm-s decreased and often became undetectable after 20 min.

In PC from Na⁺-restricted rats, amiloride-sensitive I/V relationships were recorded after the activation of apical P2R by agonists (all 10 μM; representing a maximal or near-maximal active agonist concentration); 3 min was allowed to elapse before the I/V protocol was run; see Figure 7A for a schematic of the protocol; only one measurement was made per cell). After challenging with ATP, UTP, ATPγS, or 2meSATP, Iₐm-s was found to be significantly reduced in amplitude (by 62 ± 6, 52 ± 6, 29 ± 7, and 31 ± 4%, respectively, on the basis of

### Table 2. Pharmacologic properties corresponding to P2R subtypes identified in rat CD PC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P2X₄</th>
<th>P2X₆</th>
<th>P2X₄/₆</th>
<th>P2Y₂</th>
<th>P2Y₄</th>
<th>P2Y₆</th>
<th>P2Y₁₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Agonist</td>
<td>–</td>
<td>Agonist</td>
<td>Agonist</td>
<td>Agonist</td>
<td>i.a.</td>
<td>Agonist</td>
</tr>
<tr>
<td>ATPγS</td>
<td>Agonist</td>
<td>–</td>
<td>Agonist</td>
<td>Agonist</td>
<td>Agonist (partial)</td>
<td>i.a.</td>
<td>Agonist</td>
</tr>
<tr>
<td>Ap₆A</td>
<td>i.a.</td>
<td>–</td>
<td>i.a.</td>
<td>Agonist</td>
<td>i.a.</td>
<td>i.a.</td>
<td>i.a.</td>
</tr>
<tr>
<td>UTP</td>
<td>i.a.</td>
<td>–</td>
<td>i.a.</td>
<td>Agonist</td>
<td>Agonist (partial)</td>
<td>Agonist (partial)</td>
<td>Agonist</td>
</tr>
<tr>
<td>2meSATP</td>
<td>Agonist</td>
<td>–</td>
<td>i.a.</td>
<td>Agonist (partial)</td>
<td>Agonist (partial)</td>
<td>Agonist (partial)</td>
<td>Agonist (weak)</td>
</tr>
<tr>
<td>2meSADP</td>
<td>i.a.</td>
<td>–</td>
<td>i.a.</td>
<td>i.a.</td>
<td>i.a.</td>
<td>i.a.</td>
<td>i.a.</td>
</tr>
<tr>
<td>BzATP</td>
<td>i.a.</td>
<td>–</td>
<td>i.a.</td>
<td>Agonist</td>
<td>Antagonist (weak)</td>
<td>i.a.</td>
<td>Agonist</td>
</tr>
</tbody>
</table>

*P2R were identified in our immunohistochemistry and real-time-PCR studies. Information taken from Wildman et al.⁴¹,⁴²,⁵⁹ and King and Townsend-Nicholson.⁵ Agonist, potent agonist at rat P2R orthologue; Agonist (partial), partial agonist at rat P2R orthologue; Agonist (weak), weak agonist at rat P2R orthologue where EC₁₀₀ activity would be expected ≥1 mM; i.a., inactive agonist at 10 μM; –, where the P2R subtype functions poorly as a homomeric assembly and therefore agonist activity cannot be determined.

Figure 3. P2R agonists evoke whole-cell inward currents in voltage-clamped rat CD PC in situ. (A) Typical trace showing a series of P2R agonist-evoked (10 μM) currents. The PC was voltage-clamped at −60 mV, and 3 min was left between agonist applications. (B) Currents evoked by 2meSATP were insensitive to DIDS (100 μM; preincubated for 3 min) and shown to be cationic, consistent with P2X channels. Currents evoked by Ap₆A, UTP (see B), and 2meSADP were abolished by DIDS (100 μM; preincubated for 3 min), consistent with P2Y activation of Ca²⁺−dependent Cl⁻ currents.

224 ± 38 (BzATP; n = 3; Figure 4B). Ap₆A and 2meSADP (both 10 μM) failed to evoke significant inward currents (i.e., currents ≥20 pA; n = 5). Currents evoked by the application of UTP and BzATP were abolished after preincubation (3 min) and co-application of DIDS (100 μM; n = 3 in each case; data not shown); currents evoked by 2meSATP were unaffected after DIDS treatment.

Concentration-response (C/R) curves were constructed for the range of nucleotide agonists used. As shown in Figure 5A, in Na⁺-replete rats, agonist C/R relationships demonstrated that ATPγS, UTP, 2meSATP, and BzATP are full agonists, with all but the last having similar potency to that of ATP. EC₅₀ values were as follows: ATP 1.3 ± 0.8 μM (n = 5); ATPγS 2.8 ± 1.1 μM (n = 5); UTP 2.8 ± 0.9 μM (n = 5); 2meSATP 1.3 ± 0.9 μM (n = 4); BzATP 18 ± 3 μM (n = 3). In contrast, Ap₆A was only a partial agonist and 2meSADP was inactive at concentrations up to 1 mM.
calculations using values at $-60 \text{ mV}; P < 0.01; n = 12$ in all cases), yet the $E_{\text{rev}}$ value and inward rectification did not change significantly (Figure 7B). The inward currents evoked by $\text{Ap}_6\text{A}, 2\text{meSATP}$, and $\text{BzATP}$ failed to alter significantly the amplitude or $E_{\text{rev}}$ of subsequent amiloride-sensitive I/V rela-
tions \((n = 12)\). \(E_{\text{rev}}\) values for \(I_{\text{am-s}}\) were as follows: 58 ± 7 mV (control), 57 ± 6 mV (post-ATP), 56 ± 8 mV (post-UTP), 62 ± 4 mV (post-ATP\(\gamma S\)), 58 ± 9 mV (post-2meSATP), 57 ± 6 mV (Ap\(\alpha\)A), 58 ± 5 mV (2meSADP), and 60 ± 12 mV (BzATP).

It was reported previously that extracellular Na\(^+\) ([Na\(^+\)]\(_{\text{ext}}\)) inhibits the activity of P2X receptors.\(^{23-24}\) Given that we found P2X\(_4\) subunits to be the predominant apically expressed P2XR in CD PC and our [Na\(^+\)]\(_{\text{ext}}\) levels were relatively high compared with in vivo micropuncture studies of the rat distal nephron,\(^{17}\) we next investigated the effect of decreasing [Na\(^+\)]\(_{\text{ext}}\) concentration from 145 to 50 mM (the concentration of Na\(^+\) expected in the luminal fluid of the distal tubule) on nucleotide-evoked inhibition of \(I_{\text{am-s}}\) in Na\(^+\)-restricted rats (Figure 7C). When [Na\(^+\)]\(_{\text{ext}}\) was decreased, control values for amiloride-sensitive \(I/V\) relationships were found to be significantly smaller in amplitude (decreased by 49 ± 6%, on the basis of calculations using values at −60 mV normalized to the amiloride-insensitive current; \(P < 0.05; n = 5\)), with no significant change in inward rectification or mean reversal potential. Furthermore, at this decreased [Na\(^+\)]\(_{\text{ext}}\) 2meSATP and ATP\(\gamma S\) significantly increased the amplitude of amiloride-sensitive \(I/V\) relationships by 77 ± 4 and 64 ± 7% \((P < 0.01)\) without altering inward rectification or mean reversal potential. Decreasing [Na\(^+\)]\(_{\text{ext}}\) had no significant effect on the UTP-mediated decreases in \(I_{\text{am-s}}\) amplitude but decreased the inhibitory effect of ATP from 62 ± 6% inhibition to 30 ± 9% inhibition \((P < 0.05; n = 5)\), without altering the \(E_{\text{rev}}\) or inward rectification. \(E_{\text{rev}}\) values for \(I_{\text{am-s}}\) were as follows: 53 ± 6 mV (control), 56 ± 6 mV (post-ATP), 49 ± 10 mV (post-UTP), 59 ± 6 mV (post-ATP\(\gamma S\)), 58.5 ± 9 mV (post-2meSATP), 52 ± 5 mV (Ap\(\alpha\)A), 53 ± 5 mV (2meSADP), and 52 ± 7 mV (BzATP).

When extracellular Na\(^+\) concentrations were changed from 145 to 50 mM, maximal amplitudes of DIDS-insensitive currents evoked by ATP, 2meSATP, and ATP\(\gamma S\) (100 \(\mu M\), \(V_h = −60\) mV) were significantly increased, by 11 ± 4, 32 ± 9, and 36 ± 5%, respectively \((P < 0.05; n = 5)\). In contrast, the amplitude of UTP-evoked currents did not change significantly. The C/R curves for ATP, 2meSATP, and ATP\(\gamma S\) were not significantly shifted when [Na\(^+\)]\(_{\text{ext}}\) was decreased (data not shown).

**Mechanisms of \(I_{\text{am-s}}\) Inhibition and Potentiation**

Previous studies demonstrated that activation of phospholipase C\(_\beta\) (PLC\(_\beta\)), which can be blocked by U73122, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and/or activation of phosphatidylinositol-3 kinase (PI3K; which can be blocked by wortmannin or LY294002), may be involved in P2R-mediated inhibition of ENaC activity.\(^{14,16,25,26}\) With these observations in mind, the molecular mechanisms of P2R-mediated inhibition and potentiation of \(I_{\text{am-s}}\) amplitude were investigated pharmacologically in Na\(^+\)-restricted rats. Two P2R agonists were selected for investigation, on the basis of their modulation of \(I_{\text{am-s}}\): 2meSATP (predicted in this system to target apical P2X\(_4\) and/or \(\alpha_6/\beta_3\) receptor) and UTP (predicted to target P2Y\(_2\) and/or \(\alpha_4/\beta_3\) receptor).

When P2R were activated by UTP (10 \(\mu M\)) and [Na\(^+\)]\(_{\text{ext}}\) was 145 mM, preincubation (3 min) and co-application with U73122 (100 nM) led to a significant decrease in the percent-age inhibition of \(I_{\text{am-s}}\) amplitude from 53 ± 8 to 30 ± 8% \((V_h = −60\) mV; \(P < 0.01; n = 4\); Figure 8A), with no change in the amplitude of the UTP-evoked current. Preincubation/co-application with wortmannin (100 nM), U73343 (100 nM; the inactive form of U73122), or LY294002 (50 \(\mu M\); data not shown) had no significant effect on the degree of UTP-mediated inhibition of \(I_{\text{am-s}}\) \((n = 3\) in all cases; Figure 8A). When

**Figure 8.** P2Y\(_2\)-mediated inhibition and P2XR-mediated stimulation of ENaC activity in whole-cell patch-clamped PC from Na\(^+\)-restricted rats are mediated via PLC and PI3K activation, respectively. (A) When ENaC activity is inhibited after activation of P2Y\(_2\) and/or \(\alpha_4/\beta_3\) receptor by UTP [Na\(^+\)]\(_{\text{ext}}\) = 145 mM, P2R-mediated inhibition is significantly inhibited (denoted by *) by co-application and incubation (3 min) of U73122 (100 nM; \(n = 4\); \(P < 0.01\)). (B) When ENaC activity is inhibited by 2meSATP-evoked currents (i.e., P2X\(_4\) and/or \(\alpha_6/\beta_3\) mediated), P2R-mediated inhibition is unaffected by co-application and incubation (3 min) of wortmannin or U73122 (both 100 nM; \(P < 0.01; n = 4\)). (C) As in A but where the concentration of Na\(^+\) in the perfusate is decreased to 50 mM (\(n = 3\); significance denoted by *). (D) When the concentration of Na\(^+\) in the perfusate is decreased to 50 mM, 2meSATP-evoked stimulation of ENaC activity is abolished by co-application and incubation (3 min) of wortmannin (100 nM; \(n = 4\); significance denoted by *). Data are means ± SEM.
P2R were activated by 2meSATP (10 μM), preincubation and co-application of wortmannin (100 nM), U73122 (100 nM), U73343 (100 nM), or LY294002 (50 μM; data not shown) did not significantly affect $I_{\text{am-s}}$ inhibition ($n = 3$; Figure 8B).

When $[\text{Na}^+]_{\text{ext}}$ was lowered to 50 mM, U73122 (100 nM) treatment abolished UTP-mediated inhibition of $I_{\text{am-s}}$ ($n = 3$), whereas wortmannin (100 nM), U73343 (100 nM), or LY294002 (50 μM; data not shown) had no significant effect ($n = 3$ in all cases; Figure 8C). When P2R were activated by 2meSATP (10 μM), treatment with wortmannin (100 nM) or LY294002 (50 μM; data not shown) abolished P2R-mediated increases in $I_{\text{am-s}}$ ($n = 3$), whereas U73122 (100 nM) or U73343 (100 nM) had no significant effect ($n = 4$; Figure 8D).

**DISCUSSION**

The main findings of this investigation revealed that activation of apically expressed P2R can differentially regulate ENaC activity in PC of the rat CD. More specifically, we present functional evidence that (1) activation of metabotropic P2YR (probably the P2Y$_2$ and/or P2Y$_4$ subtype) decreases $I_{\text{am-s}}$ amplitude (i.e., inhibits ENaC activity) at any $[\text{Na}^+]_{\text{ext}}$ and (2) activation of ionotropic P2XR (probably P2X$_4$ and/or P2X$_{4/6}$ subtype) can either decrease or increase the amplitude of $I_{\text{am-s}}$ (i.e., inhibit or potentiate ENaC activity), depending on $[\text{Na}^+]_{\text{ext}}$. The latter finding leads to the proposal that P2X$_4$ and/or P2X$_{4/6}$ receptor ion channels function as apically expressed Na$^+$ sensors responsible for rapid (within 3 min) and local regulation of ENaC activity in the rat CD in response to changes in luminal $[\text{Na}^+]_{\text{ext}}$.

**P2R Expression in the CD**

We characterized pharmacologically and immunohistochemically P2R expressed in PC of the cortical and outer medullary CD, in both Na$^+$-replete and Na$^+$-restricted rats. We consistently demonstrated apical expression of P2X$_4$ and $\gamma$ subunits and P2Y$_{2,4/6}$ and $\delta$ receptors in Na$^+$-replete rats and apical expression of P2X$_4$ and $\gamma$ subunits and P2Y$_{2,4/6}$ and $\delta$ receptors in Na$^+$-restricted rats in which functional ENaC was expressed (see Table 1). Furthermore, using real-time PCR, we demonstrated that dietary Na$^+$ restriction increases mRNA levels of apically expressed P2X$_4$ and $\gamma$ subunits and P2Y$_4$ receptors but not of P2Y$_2$ receptors (see Figure 2).

Our immunohistochemical findings demonstrating apical localization of P2R in rat PC are broadly in accordance with previous studies that collectively reported P2X$_4$ and $\gamma$ and P2Y$_{2,4/6}$ and $\delta$ localization/expression in native epithelia of rats, mice, and rabbits and in epithelial cell lines.6,27–39 They also extend the findings of an earlier study in which, using Na$^+$-replete rats and a limited number of commercially available P2R antibodies, we demonstrated a variety of P2R expressed throughout the nephron.40

**P2 Receptor-Mediated Inhibition of $I_{\text{am-s}}$ Amplitude**

It has already been established that the activation of apically expressed P2R by extracellular nucleotides can decrease $I_{\text{am-s}}$ amplitude in the CD,11–14,17–19 but argument remains over the P2R subtype(s) responsible. Previous pharmacologic profiling studies using mouse isolated CD reported that apical G protein–coupled P2Y$_2$-like receptors mediated ATP-evoked inhibition of ENaC activity.18,19 Furthermore, distal nephron cell studies using mouse M1 and Xenopus A6 cells suggested the exclusive involvement of an apical P2Y$_2$-like subtype.11,12,14 In contrast, in a more recent in vivo microperfusion study in the rat, we proposed an apical ionotropic ATP$_\gamma$S-sensitive P2XR-mediated effect,17 likely to be via a P2X$_{4/6}$ receptor ion channel.41 In this context, we have demonstrated that certain recombinant rat P2XR (including P2X$_4$ and P2Y$_4$) can inhibit ENaC activity in Xenopus oocytes.16 Finally, a study using mouse mIMCD-K2 cells suggested the involvement of both P2X (including P2X$_4$) and P2Y (including P2Y$_2$) subtypes.13

By using a variety of P2R agonists (and taking account of our immunohistochemistry and real-time PCR findings), this study provides evidence for both DIDS-insensitive P2XR-mediated (P2X$_4$ and/or P2X$_{4/6}$) and DIDS-sensitive P2YR-mediated (P2Y$_2$ and/or P2Y$_4$) decreases in $I_{\text{am-s}}$ amplitude in PC from rat CD. Thus, our findings are consistent with previous reports that suggested P2Y$_2$ and/or ATP$_\gamma$S-sensitive P2XR (including P2X$_4$) involvement.11–14,17–19 In addition, we suggest that additional P2R subtypes/subunits are involved (i.e., rat P2Y$_4$, which is pharmacologically similar to rat P2Y$_{2/4}$ and P2X$_{4/6}$). We have been unable to distinguish between P2X$_4$ and P2X$_{4/6}$ subtypes and between P2Y$_4$ and P2Y$_2$ receptors because of their similar pharmacologic profiles and the lack of any truly selective P2R agonists and antagonists;5,42 however, our real-time PCR studies suggested a greater P2X$_{4/6}$ and P2Y$_4$ involvement, because mRNA levels for these subunits/receptors were significantly increased after dietary Na$^+$ restriction and consequent ENaC upregulation (see Figure 2).

It is clear from our experiments that P2X-mediated inhibition (i.e., evoked by ATP$_\gamma$S or 2meSATP) is approximately 50% less than that mediated by P2Y receptors (i.e., evoked by UTP; see Figure 7A), which may explain why several studies have failed to implicate clearly an inhibitory P2X effect (i.e., the P2Y effect may mask the P2X effect).

**P2X Receptor–Mediated Potentiation of $I_{\text{am-s}}$ Amplitude**

The most striking finding of this study is that when $[\text{Na}^+]_{\text{ext}}$ was reduced (from 145 to 50 mM; one where $[\text{Na}^+]_{\text{ext}}$–mediated inhibition of P2X$_4$ and/or P2X$_{4/6}$ activity is reduced),23,24 P2X$_4$ and/or P2X$_{4/6}$ activation did not inhibit ENaC activity but potentiated it. To our knowledge this is the first report of apical P2XR potentiating ENaC activity in the CD and of a P2R differentially regulating an ion channel. A possible reason for this is that previous electrophysiologic and perfusion studies investigating the effect of P2R activation on
ENaC activity used extracellular solutions containing 145 mM Na⁺ (to optimize Na⁺ absorption).11−13,18,19 Our novel observation when using 50 mM [Na⁺]ext is important because this intraluminal Na⁺ concentration is much more physiologic (with respect to the distal nephron) than the previously used 145 mM.

The only other investigation to have used a physiologic Na⁺ concentration is a microperfusion study of the rat CD in vivo, in which a series of P2R agonists were tested intraluminally.17 In that study, a small but consistent inhibitory influence of ATPγS on CD Na⁺ reabsorption was found. It is difficult to reconcile this in vivo observation with our finding in patch-clamped rat PC that activation of P2X₄ and/or 4/6 receptors (by the application of ATPγS) caused potentiation of ENaC activity at a luminal Na⁺ concentration of 50 mM. It is conceivable that subtle differences between the experimental conditions (e.g., in the in vivo study, P2R agonists were applied for approximately 5 min and at a dosage of 1 mM) might account for the discrepancy and that in vivo inhibitory effects of P2YR activation were able to override stimulatory effects of P2XR activation, which conceivably could have been desensitized by the high concentration of agonist, although it should be noted that Shirley et al.17 found that relatively selective agonists of P2Y₂ and 4 receptors had no detectable effect on Na⁺ reabsorption.

**Mechanism of P2R-Mediated Regulation of ENaC**

As with the P2R subtype(s) responsible for inhibition of ENaC in the CD, there is also controversy over the proposed mechanism(s). Proposals have included activation of PLC14,25; decreased levels of PIP₂ in the plasma membrane as a result of PLC- and or protein kinase C−induced hydrolysis26; increases in intracellular Ca²⁺ after inositol IP₃ and diacylglycerol formation26; activation of protein kinase C after IP₃ and diacylglycerol formation26; influxes of extracellular Ca²⁺ ions26; increases in intracellular Na⁺ and downstream involvement of Gₛ proteins,16,14; activation of PI3K16; and changes in intracellular Cl⁻ levels, which in some cases involved Gₛ proteins.11,16,25

In this study, we investigated the mechanism(s) responsible for both P2R-mediated inhibition and potentiation of ENaC in PC of the CD in situ. Our data suggest the involvement of PLC activation in the inhibition of Iₛₐₐₐ by P2YR activation, irrespective of [Na⁺]ext concentration (given that P2Y-mediated decreases in Iₛₐₐₐ amplitude can be inhibited by U73122), which agrees with mechanisms already proposed.14,25,26 It is interesting that P2Y-mediated inhibition was completely abolished by U73122 when [Na⁺]ext was low but only reduced when [Na⁺]ext was high, which suggests that more than one mechanism is responsible for P2Y-mediated inhibition of ENaC when [Na⁺]ext is high (but not involving PI3K activation, because wortmannin and LY294002 have no effect; see Figure 8A).

The results with wortmannin (and LY294002) suggest the involvement of PI3K activation in the potentiation of Iₛₐₐₐ by P2X₄ and/or 4/6 activation when [Na⁺]ext was low (50 mM). In this respect, activation of PI3K by P2X₄ receptor assemblies was previously described in embryonic stem cells and microglia.44,45 When [Na⁺]ext was high (145 mM), P2X₄ and/or 4/6 activation resulted in an inhibition of Iₛₐₐₐ yet this was not altered by U73122, wortmannin, or LY294002. In contrast, when P2X₄ and/or 4/6 receptors were activated in the absence of extracellular Na⁺ (i.e., 2meSATP was prepared in a Na⁺-free Ringer solution), Iₛₐₐₐ was not inhibited (data not shown), indicating a requirement for Na⁺ influx in P2X-Mediated inhibition of ENaC. Furthermore, we showed previously that activation of P2X₄ and P2X₄/6 receptors when [Na⁺]ext was relatively high (100 mM) inhibited ENaC activity through an increase in intracellular Na⁺ and Ca²⁺,16 which may also be the case here.

**Is the P2X₄ and/or 4/6 Receptor a Na⁺ Sensor?**

The results of this study indicate that whereas apical P2Y₂ and/or 4 receptors are consistently inhibitory with respect to ENaC activity, P2X₄ and/or 4/6 receptors have the ability to inhibit or potentiate ENaC, depending on the prevailing luminal Na⁺ concentration. Under normal circumstances, where functional ENaC is sparse, the luminal Na⁺ concentration is approximately 50 mM throughout the distal tubule⁶ but may rise to approximately 100 mM along the CD. In conditions of Na⁺ restriction, although the Na⁺ concentration in the distal tubule is little affected, it falls progressively in the CD and may be as low as 3 mM in the final urine.³⁷ Under the latter condition, it would be expected that stimulation of apical P2X₄ and/or 4/6 receptors should increase apical ENaC activity and facilitate Na⁺ reabsorption in the CD (see Figure 9).

It is conceivable also that P2X₄ and/or 4/6 receptor modulation of ENaC activity might be instrumental in allowing vasopressin-mediated changes in water reabsorption to take place without corresponding changes in sodium excretion. Vasopressin itself (in physiologic concentrations) tends to reduce sodium excretion in two ways: First, by a direct stimulatory effect on sodium reabsorption in the thick ascending loop of Henle and cortical CD⁸⁴,⁹; and, second, as a result of enhanced water reabsorption in the CD and the consequent increase in intraluminal sodium concentration, which increases the driving force for sodium reabsorption. According to the findings in this study, any increase in intraluminal sodium concentration should suppress the potentiating effect of P2X₄ and/or 4/6 receptors on ENaC activity and unmask the inhibitory effect of P2Y₂ and/or 4 (and P2X₄ and/or 4/6) receptors on ENaC activity. In this way, sodium excretion could be stabilized.

It is not possible to say at present whether this P2R-mediated modulation of ENaC activity occurs by altering the channel’s apical membrane insertion or retrieval (as seen in the Xenopus oocyte expression system with regard to ENaC inhibition)⁵⁶ or by changing its open probability. What is clear is that through its action on functional ENaC activity, the apical P2R system has the potential to alter Na⁺ reabsorption significantly and in so doing may affect sodium balance and arterial BP. That hypertension is a feature of both P2X₄ and P2Y₂ knockout mice suggests an important interplay within the P2R regulatory system and sodium homeostasis in vivo.⁴⁹,⁵⁰
Animals
Adult male Sprague-Dawley rats (approximately 200 g; bred in-house) were maintained on rat chow pellets and tap water (ad libitum). Rat chow was manufactured by Special Diet Services (Witham, UK) to contain either a standard (“normal”) Na\(^+\)/H\(^+\) content (0.5% NaCl) or a low Na\(^+\)/H\(^+\) content (0.01% NaCl) intended to increase ENaC expression throughout the CD.\(^{22,52,53}\) Rats were maintained on either diet for 10 d and culled on day 11.

Antibodies
Polyclonal primary antibodies (Ab) recognizing intracellular domains of rat P2X subunit proteins were obtained from Prof. G. Burnstock (Autonomic Neuroscience Institute, UCL, London, UK; for epitope sequences, see Turner et al.\(^{40}\); P2Y\(_1\), 2, 4, 6, 11, 12, 13, and 14 receptor Ab were purchased from Alomone Laboratories (Jerusalem, Israel); \(\alpha\), \(\beta\), and \(\gamma\)-ENaC Ab were obtained from Prof. B. Rossier (University of Lausanne, Lausanne, Switzerland; for epitope sequences, see Lin et al.\(^{54}\)); and AQP2 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Noncommercially available P2X Ab were previously demonstrated to show subunit specificity.\(^{55}\)

Immunohistochemistry
Rats were terminally anesthetized by intraperitoneal injection of sodium pentobarbitone ([200 mg/ml] used at 60 mg/kg), and the kidneys were perfusion-fixed with paraformaldehyde (4%). Kidneys were removed, embedded in OCT compound (Agar Scientific, Stansted, UK), and snap-frozen. Tissues were sectioned at 8\(\mu\)m using a cryostat (Reichert Jung CM 1800, Milton Keynes, UK). The slides were stored at \(-70^\circ\)C and thawed at room temperature for 10 min before treatment.

Slide-mounted sections were washed in PBS, treated with 10% donkey serum PBS (1 h), incubated overnight at 4\(^\circ\)C with either P2R or ENaC primary Ab, and then incubated for 2 h with AQP2 primary Ab. Binding was revealed with FITC-conjugated and Cy3-conjugated (Jackson Immunoresearch Laboratories, West Grove, PA) secondary Ab. Positive AQP2 immunostaining was used to identify PC of the CD.

Slides were mounted using Citifluor mountant (Agar Scientific, Essex, UK) and examined using a Bio-Rad Radiance 2100 confocal laser scanning microscope linked to a Nikon E800 fluorescence microscope and using a ×40, 1.3 NA, oil immersion objective. Controls for nonspecific binding of

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**Figure 9.** Regulation of ENaC activity by P2R in PC of Na\(^+\)-restricted rats. ENaC activity is differentially regulated by P2X\(_4\) and/or 4/6 receptors, depending on the concentration of luminal Na\(^+\). Real-time PCR and immunohistochemistry suggest that levels of P2Y\(_4\) and P2X\(_4\) and/or 4/6 are increased when ENaC is expressed. Nucleotides have been proposed to be released from tubular cells. (A) When the concentration of luminal Na\(^+\) is low (i.e., at 50 mM in our experiments), activation of apically expressed P2X\(_4\) and/or 4/6 receptors (which are highly permeable to Ca\(^{2+}\) and, to a lesser extent, Na\(^+\) [ratio 4:1]; and in our experiments selectively targeted by 2meSATP) increases ENaC activity through the activation of PI3K. In contrast, activation (by either ATP or UTP) of apically expressed P2Y\(_2\) and/or 4 receptors inhibits ENaC activity through the activation of PLC. The overall effect of P2R activation (i.e., both P2X and P2Y, by using ATP) is a small degree of ENaC inhibition. (B) When the concentration of luminal Na\(^+\) is high (i.e., at 145 mM in our experiments), apically expressed P2X\(_4\) and/or 4/6 receptor channel activity is inhibited. In this situation, activation of P2X\(_4\) and/or 4 receptors results in inhibition of ENaC activity by an unidentified mechanism, possibly involving an influx of Na\(^+\). As before, activation of apically expressed P2Y\(_2\) and/or 4 receptor inhibits ENaC activity through the activation of PLC. The overall effect of P2R activation (i.e., both P2X and P2Y, by using ATP) is a larger degree of ENaC inhibition. We propose that P2X\(_4\) and/or 4/6 receptors act as extracellular Na\(^+\) sensors involved in the regulation of ENaC activity in PC of the rat CD.
primary and secondary Ab were performed by preincubating immune sera with their respective immunogenic peptide (overnight) or by omitting the primary Ab for histochemical procedures, respectively.

**Isolation of Tubules**

Rats were terminally anesthetized by intraperitoneal injection of sodium pentobarbitone ([200 mg/ml] used at 60 mg/kg). The kidneys were removed, decapsulated, sliced into thin corticomedullary pyramids, and placed in an ice-cold dissection solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 2 glucose, and 10 Hepes (pH 7.4, NaOH). CD from the cortical and outer medullary regions were isolated under a dissecting microscope, as described previously. Where necessary, collagenase (NB4G; 1 mg/ml for 20 min; SERVA, Heidelberg, Germany) was used on pyramids to aid tubule isolation. CD were identified by their anatomy and morphology, as described previously.

**Real-Time PCR**

RNA was extracted from pools of microdissected CD (total length 15 to 20 mm) from the cortical and outer medullary regions using an adapted guanidium thiocyanate-phenol-chloroform method. One microgram of total RNA was reverse-transcribed with 0.5 g oligo(-dt) 12 to 18 primer and a first-strand cDNA synthesis kit (Superscript II RNase H$^-$ reverse transcriptase; Life Technologies BRL, Paisley, UK). The resulting cDNA transcripts were used for PCR amplification using a Roche Lightcycler (Roche Diagnostics, Mannheim, Germany) and QuantiTect SYBR Green PCR kit (Qiagen, West Sussex UK). Gene-specific primers for P2R and the constitutively expressed gene HPRT were used (Table 3).

For quantification mRNA expression, standard curves were generated with known amounts of each PCR product. PCR products for each gene were separated on 2% (wt/vol) agarose–Tris-acetate EDTA gel containing 0.5 g/ml ethidium bromide (Sigma-Aldrich Co., Ltd., Poole, UK). PCR bands were observed under ultraviolet illumination, excised from the gel, and purified using a Geneclean kit (Qbiogene, Cambridge, UK). Purified DNA was serially diluted 10-fold covering a dynamic range of 6 logarithmic orders, and 1 l of each standard was amplified by PCR using the relevant gene-specific primers. One set of P2R standards was amplified in duplicate with the HPRT standards to generate two standard curves. For each sample, a ratio of relative abundance of each gene to the housekeeping gene HPRT was calculated by the Lightcycler Relative Quantification software, Version 1.0 (Roche Diagnostics). Melting curve analysis was carried out to determine primer specificity. PCR products were also analyzed by gel electrophoresis and visualized using a Bio-Rad multi-imager (Bio-Rad, Hemel Hempstead, UK).

**Flame Photometry**

Upon removal of the kidneys, urine was collected from the bladder and the Na$^+$ content was measured by flame photometry (model 543; Instrumentation Laboratory, Warrington, UK) to confirm that reduced dietary Na$^+$ intake decreased urinary Na$^+$ content.

**Patch Clamp**

Isolated tubules were immobilized on glass coverslips coated with poly-L-lysine (50 g/ml in dissection solution) and transferred to a chamber (volume approximately 600 l) mounted on the stage of an inverted microscope (Nikon Eclipse TE300). CD were opened with a sharpened micropipette to access the apical membrane. PC were identified by their flatness, abundance, and polygonal shape.

The whole-cell configuration of the patch-clamp technique was used;

### Table 3. Primer sequences and expected product sizes

<table>
<thead>
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<th>Gene</th>
<th>Accession No.</th>
<th>5’ to 3’ Sequence</th>
<th>Size (bp)</th>
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<tr>
<td>Rat P2X$_1$</td>
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<td>S: GAAGTGTGATCTGGACTGGCACGT AS: GCGTCAAGTCCGGATCTCGACTAA</td>
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<td>S: TGGGCTTCTGGGATTAAGCTGGG AS: CAGTTGGCCTGGTCATGCCGA</td>
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<td>NM_080780</td>
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$^a$Primer sequences taken from Turner et al., Katugampola and Burnstock, and Shibuya et al. S, sense; AS, antisense.
this was achieved through a nystatin perforated-patch method. For establishment of the perforated-patch, nystatin (50 to 100 μg/ml) was added to the pipette solution containing (in mM) 50 KCl, 5 K-gluconate, 3 MgCl₂, 3 EGTA, and 10 Hepes (pH 7.2, KOH). The development of electrical access to the cell interior was monitored by following the nystatin-induced fall in access resistance (Rᵢ). Experiments were initiated once Rᵢ had fallen to a stable value of <35 MΩ (this typically took approximately 10 min) and subsequent series resistance compensation was applied. An Axopatch 200B patch-clamp amplifier (Axon Instruments, Sunnyvale, CA) and computer complete with pCLAMP 8.0 software (Axon Instruments) were used to store and analyze whole-cell currents. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) and had a resistance ranging between 4.0 and 6.0 MΩ. The reference electrode was an Ag/AgCl pellet. Current signals were filtered at 5 kHz and displayed on an oscilloscope (20 MHz digital storage; Gould, Eichstetten, Germany). Liquid junction potentials were calculated using pCLAMP software, and appropriate corrections were applied.

Split-open CD were slowly superfused (4 ml/min, low enough to avoid mechanical disturbances and associated endogenous ATP release; 21 to 23°C) with bathing solution that contained (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 Hepes (pH 7.4, NaOH) by a gravity-fed, continuous-flow system that allowed drug addition and washout. In experiments to assess the effect of altering [Na⁺]ₑₓₑₜ, Rᵢ was altered to contain (in mM) 45 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 Hepes, 90 TEA-Cl, and 10 NMDG⁺ (pH 7.4, NaOH). P2R agonist-activated membrane currents were recorded at a Vₑ of −60 mV, sufficient to drive P2X-mediated inward cationic currents and P2Y-mediated Ca²⁺-dependent chloride currents. P2R agonists were applied for 30 s or until the current reached a peak, whichever was the longer, then washed out for 3 min to avoid rundown. Only cells that demonstrated an inward current amplitude (evoked by 10 μM ATP) >100 pA were used. Aminolide-sensitive whole-cell currents (Iₐₛₛ) were calculated as the difference between total currents and currents obtained in the presence of aminolide (10 μM). Steady-state I/V relations were determined for each whole-cell clamp by stepping the command voltage down to −60 mV and up to 80 mV from 0 mV in 20-mV increments for 100 ms.

Statistical Analyses
All data are presented as mean ± SEM; significance was evaluated by t test, paired or unpaired as appropriate (Instat 3.0; GraphPad Software, San Diego, CA), with P < 0.05 considered significant.

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DISCLOSURES
None.

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