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Nucleotides Downregulate Aquaporin 2 via Activation of Apical P2 Receptors

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ABSTRACT
Vasopressin regulates water reabsorption in the collecting duct, but extracellular nucleotides modulate this regulation through incompletely understood mechanisms. We investigated these mechanisms using immortalized mouse collecting duct (mpkCCD) cells. Basolateral exposure to dDAVP induced AQP2 localization to the apical membrane, but co-treatment with ATP internalized AQP2. Because plasma membrane-bound P2 receptors (P2R) mediate the effects of extracellular nucleotides, we examined the abundance and localization of P2R in mpkCCD cells. In the absence of dDAVP, P2Y1 and P2Y4 receptors localized to the apical membrane, whereas P2X2, P2X4, P2X5, P2X7, P2Y2, P2Y11, and P2Y12 receptors localized to the cytoplasm. dDAVP induced gene expression of P2X1, which localized to the apical domain, and led to translocation of P2X2 and P2Y2 to the apical and basolateral membranes, respectively. In co-expression experiments, P2R activation decreased membrane AQP2 and AQP2-mediated water permeability in Xenopus oocytes expressing P2X2, P2Y2, or P2Y4 receptors, but not in oocytes expressing other P2R subtypes. In summary, these data suggest that AQP2-mediated water transport is downregulated not only by basolateral nucleotides, mediated by P2Y2 receptors, but also by luminal nucleotides, mediated by P2X2 and/or P2Y4 receptors.


Urinary concentration and water homeostasis are primarily under the control of arginine vasopressin (AVP) acting on the renal collecting duct (CD). AVP ultimately exerts its effect by controlling the abundance of aquaporin 2 (AQP2) water channels in the apical membrane of CD principal cells (PCs): it activates V2 receptors in the basolateral membrane of these cells and stimulates adenyl cyclase (increasing intracellular cAMP levels), which in turn results in phosphorylation of AQP2 and its rapid (within minutes) trafficking and insertion into the apical membrane (from subapical storage vesicles),1,2 together with reduced endocytosis of AQP2;3 continued exposure to AVP over several days stimulates AQP2 gene transcription.4,5

Increased apical abundance of AQP2 enhances water reabsorption and produces a concentrated urine; in the absence of AVP, AQP2 is internalized by ubiquitin-dependent endocytosis,6 leading to reduced water reabsorption and a dilute urine. Therefore, any dysregulation of AQP2 synthesis or sur-
face membrane abundance will affect fluid balance: lack of normal AQP2 abundance or function in cranial or nephrogenic diabetes insipidus causes excessive water loss and hypernatremia,7,8,9 while increased apical membrane abundance of AQP2 in congestive heart failure, liver cirrhosis, or the syndrome of inappropriate ADH secretion causes enhanced water retention and hyponatremia.10,11

Several paracrine or autocrine factors, including extracellular nucleotides, have been shown to inhibit AVP-stimulated and AQP2-mediated water transport in the CD.12–20 The effect of extracellular nucleotides is mediated by activation of plasma membrane-bound P2 receptors (P2R) in CD PCs. The presence of almost all of the molecularly identified subtypes of ionotropic (P2X) and metabotropic (P2Y) P2R has been demonstrated in CD epithelial cells and immortalized distal nephron epithelial cell lines.21–24 Ionotropic P2X receptors are represented by homo-meric assemblies (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7) and heteromeric assemblies (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6, and P2X4/7).25 Metabotropic P2Y receptors are represented by P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y12, and the recently cloned P2Y13 and P2Y14, subtypes.25

Based on pharmacologic profiling (although it should be noted that truly specific agonists are not available for P2R), the P2R subtype thought to inhibit AVP-stimulated water transport in the CD is the P2Y2 receptor present in the basolateral membrane of PCs.12,15 This P2R-mediated inhibition is PKC-dependent, and the result of decreased intracellular cAMP and increased PGE2 levels.26,27 In support of a role for the P2Y2 receptor in modulating AVP-mediated changes in CD water transport, infusion of AVP (for 90 min) increases P2Y2 mRNA and protein levels in the CD of hydrated rats.28 Therefore, it has been proposed that activation of basolaterally located P2Y2 receptors by extracellular nucleotides significantly affects AQP2 abundance, and thereby AVP-mediated water reabsorption, in the CD.29 Indeed, P2Y2 knockout mice exhibit higher levels of AQP2 compared with wild-type mice, despite similar AVP levels.30,31

Activation of basolaterally localized P2Y2 receptors has also been linked to the local control of amiloride-sensitive Na+ reabsorption in CD PCs,22 an effect that also occurs with luminal (apical) ATP. However, we have recently shown that the P2Y2 subtype is not the only P2R to mediate a luminal effect of ATP on CD Na+ transport.24 Combining molecular and pharmacologic approaches, we found that activation of P2Y4 (which is almost identical in its pharmacologic profile to P2Y2), P2X6, or P2X4/6 receptors can also affect Na+ transport. Furthermore, we showed that changing dietary Na+ intake alters P2R mRNA and protein levels.24

Two questions arise from the previously published work: (1) Can AVP alter the protein abundance and mRNA levels of P2R other than the P2Y2 subtype? (2) Can P2R other than the P2Y2 subtype affect AVP-stimulated AQP2 abundance in CD PCs? In the present study, we have used immortalized mouse mpkCCD(c14) cells and the Xenopus oocyte heterologous expression system to clarify the molecular interactions between P2R subtypes and AQP2 abundance, and internalization.

RESULTS

Immunocytochemical Localization of AQP2 in mpkCCD Cells

To determine whether AVP induces plasma membrane localization of AQP2 in mpkCCD cells, monolayers of these cells were either left untreated or were treated with dDAVP. In the absence of dDAVP (n = 12), no immunofluorescent staining for AQP2 was detectable in the cells or their apical membranes (Figure 1A); however, treatment with dDAVP (1 nM applied to the basolateral medium for 96 h; n = 12) resulted in strongly positive immunofluorescence for AQP2 in the apical membrane (Figure 1B). Thus, as in earlier published studies,32,33

Figure 1. dDAVP-induced localization of AQP2 in mpkCCD cells. Confocal microscopy was used to visualize the cellular distribution of AQP2 in monolayers of mpkCCD cells grown to confluence on transwell permeable supports in the absence or presence of 1 nM dDAVP (applied to the basolateral medium) for 96 h. Images shown are x–y planes (large rectangle) and x–z planes (shown beneath the x–y plane). Monolayers were imaged in x–z planes from apical to basal boundaries of the cells. The x–z image is from the site indicated by the dashed line. (A) In cells untreated with dDAVP, immunostaining for AQP2 was not evident. (B) Treatment of cells with dDAVP resulted in positive immunostaining (FITC) for AQP2 in the apical domain. Scale bar represents 5 μm.
treatment with dDAVP for 96 h is sufficient to induce endogenous AQP2 synthesis and apical localization.

**mRNA Levels of P2R in mpkCCD Cells**

To investigate the relative abundance of P2R mRNA in monolayers of cells treated or untreated with dDAVP, we performed real-time PCR analysis and calculated the ratio of expression of the P2R gene of interest to a constitutively expressed housekeeping gene (hypoxanthine phosphoribosyl transferase [HPRT]). In untreated cells, we could not detect significant levels of P2X1, P2X2, or P2X7 subunit mRNA, or of P2Y6 receptor mRNA (n = 6; Figure 2A and B, respectively). In contrast, significant amounts of mRNA were detected for P2X2, P2X3, P2X6, and P2X7 subunits (Figure 2A), and for P2Y1, P2Y2, and P2Y4 receptors (Figure 2B); mRNA abundance had a rank order of P2Y2 > P2X2 = P2X4 = P2Y1 = P2Y4 > P2X3 = P2X7.

Cells treated with dDAVP showed a significant increase in abundance of P2Y2 mRNA (by 7-fold; n = 6; P < 0.01), a significant decrease in abundance of P2Y7 mRNA (by 2.5-fold; n = 6; P < 0.01), but no change in P2X2, P2X4, P2X6, or P2Y4 mRNA levels (Figure 2, A and B). P2X2 mRNA levels increased significantly in cells treated with dDAVP (Figure 2A). Rank order of abundance for P2R mRNA in cells treated with dDAVP was P2Y2 > P2X1 = P2X4 > P2X7 = P2Y1 = P2Y4 > P2X3 = P2X7.

These data suggest that mpkCCD cells express a variety of P2R and that chronic dDAVP treatment alters the mRNA levels of several P2R subtypes.

**Localization of P2R in mpkCCD Cells**

To investigate P2R protein localization in untreated mpkCCD cells, monolayers of cells (n = 6) were stained for different P2R using specific antibodies. Positive immunofluorescence for P2Y1 and P2Y4 receptors was seen apically, whereas P2X2, P2X4, P2X6, and P2X7 receptor subunits and P2Y6, P2Y11, and P2Y12 receptors were located throughout the cytoplasm, toward the cell borders and in the perinuclear region (Figure 3A). Immunofluorescent staining for P2X1, P2X3, and P2X6 subunits and the P2X4 receptor was not seen. These data broadly agree with the real-time PCR findings.

To corroborate the apical localization of P2Y1 and P2Y4 receptors in cells untreated with dDAVP, whole-cell perforated patch-clamp recordings were made on individual cells using P2R-selective agonists. Figure 3B shows ATP- and other P2R agonist-evoked (all at 10 μM) whole-cell inward currents in voltage-clamped (holding potential [Vh] = −60 mV) cells in a confluent monolayer. Agonists were chosen according to their P2R selectivity (see Table 1).24-34 The amplitudes of 10 μM agonist-evoked currents were 584 ± 59 pA (for ATP; n = 5), 343 ± 29 pA (for ATPγS; n = 5), and 234 ± 40 pA (for 2meSADP; n = 5); consistent with apical P2Y1 and P2Y4 receptor localization.

To test whether dDAVP alters the abundance and/or localization of P2R, mpkCCD cells were treated with dDAVP and subjected to immunocytochemistry using P2R-specific antibodies. Treatment with dDAVP (1 nM; 96 h; basolateral medium) resulted in positive immunostaining for P2X1 and P2X2 receptor subunits in the apical regions, and basolateral staining for P2Y2 receptors (Figure 4, A and B), suggesting that dDAVP induces the synthesis of P2X1, and targets P2X1, and P2X2 receptor subunits, as well as P2Y2, receptors, to the plasma membrane. Fluorescence immunostaining for all other P2R remained unchanged (data not shown).

To investigate whether dDAVP-dependent P2R synthesis and localization is cAMP-dependent, dDAVP-treated mpkCCD cells were incubated with the cell-permeable PKA inhib-
itor H-89 and subjected to immunocytochemistry using P2X1-, P2X2-, and P2Y2-specific antibodies. Treatment with H-89 (1 μM; 30 min; basolateral medium) abolished dDAVP-dependent P2X1 synthesis (and apical localization), dDAVP-dependent P2X2 apical localization, and dDAVP-dependent P2Y2 basolateral localization (data not shown), strongly suggesting that dDAVP-dependent P2R synthesis and localization in mpkCCD cells is cAMP-dependent. In the absence of dDAVP, H-89 failed to alter fluorescence immunostaining for any P2R.

Acute Effects of P2R Activation on AQP2 Membrane Localization in mpkCCD Cells

To investigate the effect of ATP on AQP2 localization, dDAVP-treated mpkCCD cell monolayers were treated with 1 μM ATPγS (a stable analog of ATP with agonist activity at most P2R) for 2 h, added to either the apical or basolateral medium (n = 5). Immunostaining revealed that whereas AQP2 was localized to the apical membrane in the absence of ATPγS, it was present intracellularly following exposure to ATPγS in either the apical or basolateral medium (Figure 5A), consistent with AQP2 internalization.

To confirm that ATP itself causes internalization of AQP2, ATP was added to both apical and basolateral medium of cultured mpkCCD cells or to each medium separately for 2 h. Subsequent cell surface biotinylation, and semiquantification of AQP2 abundance by immunoblotting, showed that AQP2 was internalized from the plasma membrane when ATP was added to either the apical or basolateral medium (Figure 5, B and C), and that this effect was increased when ATP was added to both medium (Figure 5, B and C). These data indicate that both apical and basolateral P2R can affect apical membrane abundance of AQP2, and that the effect is additive.

To investigate which of the apically localized P2R (P2X1, P2X2, P2Y1 and/or P2Y4; see Figures 3, A and B, and 4) causes internalization of AQP2, a selection of partially selective P2R agonists was added to the apical medium of cultured mpkCCD cells for 2 h. Subsequent cell surface biotinylation, and semiquantification of AQP2 abundance by immunoblotting, showed that AQP2 was internalized from the plasma membrane when ATP (100 μM; a potent agonist at P2X1, P2X2, and P2Y4 receptor subtypes) or UTP (100 μM; an agonist selectively favoring the P2Y4 receptor subtype), but not 2meSADP (100 μM; an agonist selective for the P2Y1 receptor subtype), was added (Figure 5B). AQP2 membrane abundance was reduced to a greater extent by ATP than by UTP. These data indicate that apical P2Y4 receptors and P2X1 and/or P2X2 receptors, but not P2Y1, play an important role in altering AQP2 abundance and localization.

Longer-term Effects of P2R activation on AQP2 Levels in mpkCCD Cells

To test whether P2R activation results in AQP2 protein degradation, dDAVP-treated mpkCCD cells were incubated with ATP (100 μM) added to both sides (apical and basolateral) for 8 h. Subsequent immunoblotting for AQP2 and β-actin (to normalize for the amount of protein loaded) indicated that AQP2 abundance decreased to a greater extent by ATP than by UTP. These data suggest that P2R activation, particularly P2X1 and P2X2, play an important role in altering AQP2 abundance and localization.

Table 1. Pharmacologic profiles of those P2R subtypes identified in the apical membrane of mpkCCD cells (in the absence of dDAVP treatment; see Figure 3)

<table>
<thead>
<tr>
<th>P2R</th>
<th>ATP</th>
<th>UTP</th>
<th>ADP</th>
<th>BzATP</th>
<th>ATPγS</th>
<th>2meSADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>agonist</td>
<td>i.a.</td>
<td>agonist</td>
<td>i.a.</td>
<td>agonist</td>
<td>agonist</td>
</tr>
<tr>
<td>P2Y4</td>
<td>agonist</td>
<td>agonist</td>
<td>i.a.</td>
<td>antagonist</td>
<td>agonist (partial)</td>
<td>i.a.</td>
</tr>
</tbody>
</table>

Information taken from King and Townsend-Nicholson, 2003; Wildman et al., 2008.24,34 i.a., inactive agonist at 10 μM.
ATP treatment reduced AQP2 protein levels by approximately 90% (Figure 6,A and B).

To investigate whether adenosine, derived from the breakdown of ATP, could be responsible for the ATP-evoked reduction in AQP2 membrane abundance, dDAVP-treated cells were incubated with ATP (100 μM) and DPCPX (10 μM; a nonselective adenosine, P1, receptor antagonist) added to both apical and basolateral sides for 8 h. Subsequent immunoblotting for AQP2 demonstrated that simultaneous treatment with ATP and DPCPX also markedly reduced AQP2 protein levels (Figure 6, C and D). Furthermore, dDAVP-treated cells were incubated with ATPγS (100 μM; a stable analog of ATP with agonist activity at most P2R) added to both sides for 8 h; subsequent immunoblotting for AQP2 demonstrated that ATPγS reduced AQP2 levels.
protein levels to a similar degree as that seen for ATP (data not shown). These findings suggest that adenosine is unlikely to be responsible for the reduction in AQP2 membrane abundance observed with ATP.

**Effect of P2R Activation on AQP2-Mediated Water Permeability in Xenopus Oocytes**

To help determine which of the P2R localized in mpkCCD cells could mediate the observed downregulation of AQP2 abundance, the P2R localized in the plasma membrane of mpkCCD cells with or without dDAVP (and a number of others) were co-expressed with AQP2 in Xenopus oocytes, and the oocytes subjected to a cell swelling assay with or without P2R activation with 10 μM ATP.

AQP2-mediated water permeability ($P_f$) was significantly decreased following P2R activation in oocytes co-expressing P2X$_2$ (by 46 ± 8%; n = 6; $P < 0.01$; Figure 7A), P2Y$_2$ (by 53 ± 7%; n = 6; $P < 0.01$; Figure 7B) or P2Y$_4$ receptors (by 57 ± 3%; n = 6; $P < 0.01$; Figure 7B). Inhibition of AQP2-mediated $P_f$ was achieved within 15 min. All other P2R tested, including P2X$_1$, did not change AQP2-mediated $P_f$ (n = 6; Figure 7, A and B).

To determine if the reduction of AQP2-mediated water permeability by P2R activation also reduced plasma membrane AQP2 abundance, total membrane and plasma membrane fractions were made. Immunoblot analysis demonstrated that in Xenopus oocytes expressing only AQP2 (Figure 8A), or AQP2 co-expressed with P2X$_1$, P2X$_3$, or P2X$_4$ receptors, extracellular ATP did not alter AQP2 membrane abundance (data not shown). In contrast, in oocytes expressing those P2R that reduced AQP2-mediated water permeability on exposure to ATP (P2X$_2$, P2Y$_2$, and P2Y$_4$), plasma membrane abundance of
AQP2 was reduced. This is consistent with P2R-mediated inhibition of AQP2 protein from the plasma membrane (n = 3; Figure 8B).

DISCUSSION

The main findings of our in vitro study in mpkCCD cells are that AVP can alter P2R abundance and localization, and that activation of apically (and basolaterally) localized P2R can cause the internalization (and degradation) of AQP2 (Figure 9). More specifically, we report that: (1) P2Y1 and P2Y4 receptors are localized in the apical membrane, independent of the presence of dDAVP; (2) AVP induces cAMP-dependent synthesis and apical localization of AQP2, and of the P2X1 receptor; (3) AVP induces cAMP-dependent translocation and subsequent localization of P2X2 and P2Y2 receptors in the apical and basolateral membrane, respectively; (4) activation of basolaterally localized P2Y2 receptors, and of apically localized P2X2, and P2Y4 receptors, stimulates AQP2 internalization in the presence of AVP. These findings suggest a complex regulatory relationship between apical and basolateral P2R in AVP-stimulated, AQP2-mediated water transport in the CD.
P2R Abundance and Localization in mpkCCD Cells
As already mentioned, in cells untreated with AVP, we demonstrated localization of P2Y1 and P2Y2 receptors in the apical membrane (corroborated by pharmacologic characterization); and of P2X1, P2X4, P2X5, and P2X7 subunits and P2Y2, P2Y11, and P2Y2 receptors throughout the cytoplasm. In cells treated with AVP, in which AQP2 is localized in the apical membrane, we demonstrated apical localization of P2X1 and P2X2 subunits, and of P2Y1 and P2Y4 receptors, and basolateral localization of P2Y2 receptors; while P2X4, P2X5, and P2X7 subunits and P2Y11 and P2Y12 receptors remained within the cytoplasm. Furthermore, using real-time PCR, we showed that AVP treatment increases mRNA levels of apically localized P2X1 and basolaterally localized P2Y2 receptors (Figure 2). Taken together, these data suggest that AVP induces not only AQP2 translocation and abundance, but also P2X1, subunit and P2Y2 receptor synthesis and membrane trafficking, and P2X2 subunit membrane trafficking.

Our immunocytochemistry findings of P2R localization in mpkCCD cells agree broadly with previous reports of the presence of P2X1, P2X5, P2X6, P2X7, and P2X8, subunits and P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, and P2Y13 receptors in rodent CD PCs.12,24,35–39 Although others have not reported P2Y1, P2X1, P2X4 and P2X6 subunits, and P2Y4 receptors, were up-regulated by low Na+ diet, an effect we attributed to an increase circulating aldosterone levels. Activation of the apically localized P2Y2 receptor inhibits the CD epithelial Na+ channel (ENaC), whereas the apical P2X2 subunit can inhibit or stimulate this channel. For the apical P2Y2 receptor at least, this suggests the possibility of a coupling between its inhibitory effect on Na+ and water transport in the CD. Interestingly, Schafer and Chen have reported that in Na+ -depleted rats the stimulatory effect of AVP on Na+ and water transport in the CD is significantly reduced.41

P2R-mediated Internalization of AQP2
Until recently, most attention in the CD has focused on P2Y2 receptors, reinforced by recent studies of a P2Y2 knockout mouse,31 and it has already been established that activation of basolaterally localized P2Y2 receptors by extracellular nucleotides can inhibit AQP2-mediated water transport in the CD.12–15 This inhibition of AVP-stimulated, AQP2-mediated water transport is PLC- and PKC-dependent, and results in decreased intracellular cAMP and increased PGE2,12,13,15,42 which is known to cause AQP2 internalization.43

The novel data provided by the present study are the effects of activating apically localized P2R, including P2X subtypes, on AQP2 trafficking. Although a previous investigation has reported that stimulation of apical P2Y2 receptors in the terminal segment of the inner medullary CD is without significant effect on AQP2-mediated water reabsorption,15 an effect by other apical P2R subtypes was not excluded in that study. Here we report that activation of apically localized P2X2 and P2X4 receptors, rather than apical P2Y2, stimulates AQP2 internalization in a cell line derived from the cortical CD, the region of the CD in which most AVP-stimulated water reabsorption occurs. It is likely that the signal transduction pathway for P2X4-mediated inhibition of AVP-sensitive water reabsorption is similar to that for P2Y2, and is Gs protein-coupled. However, from our recent study of apical P2R control of Na+ transport, we know that P2X signal transduction involves stimulation of PI3K,24 which when activated has also been shown to stimulate retrieval of AQP2 from the apical membrane.44

Physiologic Significance
Trying to establish a physiologic role for paracrine or autocrine regulators of AVP-sensitive water transport is not easy and risks unwarranted speculation. While there are obvious limitations to both in vitro and in vivo models when it comes to manipulating the levels and action of AVP, even knockout mice have so far served only to illustrate how much complex interaction and compensation can occur in the local and intracellular control of AVP-dependent water permeability in the CD.42 However, there are at least two obvious questions to ask: (1) Where does the ATP come from in the CD and how is it released? (2) Why should there be apical as well as basolateral control by P2R? Concerning the source and release of ATP, it is generally believed that ATP is released locally, and that the stimulus to release may be cell swelling or shear stress, including flow-triggered release via the PC apical membrane cili um.42,45 Cell swelling under local and more generalized hypotonic conditions, for example, in hyponatremia, could lead to ATP release, which would then help to maximize water excretion by its action on basolateral P2Y2 receptors and apical P2R. Similarly, the water diuresis in this setting could further stimulate flow-dependent ATP release to increase water loss.
In summary, while acknowledging that the present findings are from an \textit{in vitro} model, we have confirmed the location and function of the basolateral P2Y\textsubscript{2} receptor in mediating ATP inhibition of AVP-dependent water transport along the CD, but in addition, as with Na\textsuperscript{+} transport, we have defined novel P2R-mediated inhibition of AVP-dependent water transport through apically located P2R. This apical regulation involves P2X\textsubscript{2} subunits and P2Y\textsubscript{4} (rather than P2Y\textsubscript{2}) receptors; in addition, AVP itself increases the abundance and membrane trafficking of these inhibitory P2R.

**CONCISE METHODS**

**Cell Culture**

mpkCCD cells (clone 14) were grown in a modified cell medium (DMEM:Ham’s F12 1:1 vol/vol; 60 nM sodium selenate, 5 \(\mu g/ml\) transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml EGF, 5 \(\mu g/ml\) insulin, 20 mM D-glucose, 2\% FCS, and 20 mM HEPES [pH 7.4]). Cells were seeded at a density of \((1.5 \times 10^5)\) cells/cm\(^2\) on semipermeable filters (Transwell\textsuperscript{\textregistered}, 0.4\% pore size, Corning Costar, Cambridge, USA); 1.13 cm\(^2\) filters were used for immunocytochemistry or immunoblotting, and 4.7 cm\(^2\) filters for biotinylation experiments. The cells remained in culture for 8 d before being analyzed. Where stated, cells were treated with 1 nM dDAVP (added to the basolateral medium) for the last 4 d to induce AQP2 expression maximally (75 to 85\% of mpkCCD cells expressed AQP2 after dDAVP treatment),\textsuperscript{32} and the nucleotide compounds ATP\textsubscript{S}, ATP or UTP, or the PKA inhibitor H-89, were applied to the basal and/or apical medium for the final 2 h. In some cases, DPCPX (and ATP\textsubscript{S}) was applied to the basal and apical medium for the final 8 h.

**Real-Time PCR**

RNA was extracted from confluent monolayers of mpkCCD cells using an adapted guanidium thiocyanate-phenol-chloroform method. One microgram of total RNA was reverse transcribed with 0.5 \(\mu g\) oligo(-dT) 12 to 18 primer and a first-strand cDNA synthesis kit (Superscript II RNase H\textsuperscript{-} reverse transcriptase, Life Technologies BRL, UK). The resulting cDNA transcripts were used for PCR amplification using a Roche Lightcycler (Roche diagnostics, Germany) and QuantiTect SYBR\textsuperscript{®} Green PCR kit (Qiagen, West Sussex, UK). Gene-specific primers for P2R and the constitutively expressed gene hypoxanthine phosphoribosyl transferase (HPRT) were used as described previously.\textsuperscript{24} To quantify mRNA expression, standard curves were generated with known amounts of each PCR product. PCR products for each gene were separated on 2\% (w/v) agarose-TAE (Tris-acetate EDTA) gel containing 0.5 \(\mu g/ml\) 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 \(\mu g\) 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, Germany). 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).

**Immunocytochemistry**

Immunocytochemistry and confocal laser scanning microscopy of cells grown on semipermeable filters were performed as described previously.\textsuperscript{46} Cells were incubated with affinity-purified rabbit anti-AQP2 antibodies or rabbit anti-P2R antibodies.\textsuperscript{24,47} Noncommercial P2X antibodies were previously demonstrated to show subunit specificity.\textsuperscript{48}

**Patch Clamp Electrophysiology**

Patch clamp electrophysiology of cells grown on glass coverslips was performed as described previously.\textsuperscript{24} The whole-cell configuration was used; this was achieved using a nystatin perforated-patch method. To establish the perforated-patch, nystatin (50 to 100 \(\mu g/ml\)) was added to the pipette solution (50 mM KCl, 90 mM K-glucanate, 3 mM MgCl\(_2\), 3 mM EGTA and 10 mM Hepes [pH 7.2, KOH]). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus Ltd, Edenbridge, UK) and had a resistance ranging between 4.0 and 6.0 M\textOmega{}.

Cells were superfused (4 ml/min) with bathing solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\) and 10 mM Hepes [pH 7.4, NaOH]) by a gravity-fed, continuous flow system that allowed drug addition and washout. P2R agonist-activated membrane currents were recorded at a holding potential \((V_{o})\) of \(-60\) mV, sufficient to drive P2X-mediated inward cationic currents and P2Y-mediated reporter currents. P2R agonists were applied for \(60\) s or until the current reached a peak, whichever was longer, then washed out for 3 min to allow rundown.

**Water Permeability (P\textsubscript{f}) Measurements**

	extit{Xenopus laevis} oocytes were obtained and defolliculated as described previously.\textsuperscript{49} Defolliculated oocytes were injected with 50 nl of sterile water, or cRNA/cRNA combination (50\% P2R/50\% AQP2) in the case of co-expression studies (total cRNA quantities: human AQP2, 0.5 ng; rat P2X\(_1\), P2X\(_3\), P2X\(_6\), P2Y\(_1\), P2Y\(_2\), P2Y\(_4\) and P2Y\(_{6}\), 50 ng; rat P2X\(_{3}\) and P2X\(_{7}\), 20 ng). Injected oocytes were incubated for 48 h at 18 °C in Barth’s solution (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO\(_4\), 0.33 mM Ca(NO\(_3\))\(_2\), 2.4 mM NaHCO\(_3\), 0.41 mM CaCl\(_2\) and 10 mM Hepes [pH 7.4]) and was supplemented with 50 \(\mu g/L\) gentamicin sulfate, then kept at 4 °C until used in experiments. Recombinant cRNA was not available for P2Y\(_{6}\), P2Y\(_{11}\), or P2Y\(_{12}\) receptors.

Oocytes were analyzed after 48 h in a swelling assay as described previously.\textsuperscript{7} Oocyte swelling was performed at 22 °C after transfer.
from 200 to 70 mOsM/kg. Where stated, oocytes were preincubated with ATP (10 μM) for 15 min before the swelling assay started.

Immunoblotting
Immunoblotting and biotinylation experiments were performed as described previously.\(^5,7\) Affinity-purified rabbit anti-AQP2 antibodies (1:3000)\(^2\) or mouse anti-β-actin (Sigma, St. Louis, MO; 1:25,000) primary antibodies were used. As secondary antibodies, goat anti-rabbit or sheep anti-mouse antibodies coupled to horseradish peroxidase (HRP; Sigma, St. Louis, MO; 1:10,000) were used. Films were scanned using a GS-690 Imaging Densitometer (Bio-Rad, Hercules, CA) and analyzed using Bio-Rad software.

Statistical Analysis
All data are presented as mean values ± S.E.M.; significance was evaluated by t test (Instat v3.0: GraphPad Software, San Diego, CA), with P < 0.05 considered significant. Each experiment was repeated at least three times.

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DISCLOSURES
None.

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