

# 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, P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors localized to the apical membrane, whereas P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>7</sub>, P2Y<sub>2</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub> receptors localized to the cytoplasm. dDAVP induced gene expression of P2X<sub>1</sub>, which localized to the apical domain, and led to translocation of P2X<sub>2</sub> and P2Y<sub>2</sub> 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 P2X<sub>2</sub>, P2Y<sub>2</sub>, or P2Y<sub>4</sub> 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 P2Y<sub>2</sub> receptors, but also by luminal nucleotides, mediated by P2X<sub>2</sub> and/or P2Y<sub>4</sub> receptors.

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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 adenylyl 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),<sup>1,2</sup> together with reduced endocytosis of AQP2;<sup>3</sup> continued exposure to AVP over several days stimulates AQP2 gene transcription.<sup>4,5</sup>

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,<sup>6</sup> leading to reduced water reabsorption and a dilute urine. Therefore, any dysregulation of AQP2 synthesis or sur-

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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,<sup>7,8,9</sup> 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.<sup>10,11</sup>

Several paracrine or autocrine factors, including extracellular nucleotides, have been shown to inhibit AVP-stimulated and AQP2-mediated water transport in the CD.<sup>12–20</sup> 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.<sup>21–24</sup> Ionotropic P2X receptors are represented by homomeric assemblies (P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>6</sub>, and P2X<sub>7</sub>) and heteromeric assemblies (P2X<sub>1/2</sub>, P2X<sub>1/4</sub>, P2X<sub>1/5</sub>, P2X<sub>2/3</sub>, P2X<sub>2/6</sub>, P2X<sub>4/6</sub>, and P2X<sub>4/7</sub>).<sup>25</sup> Metabotropic P2Y receptors are represented by P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub>, and the recently cloned P2Y<sub>13</sub> and P2Y<sub>14</sub> subtypes.<sup>25</sup>

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 P2Y<sub>2</sub> receptor present in the basolateral membrane of PCs.<sup>12,15</sup> This P2R-mediated inhibition is PKC-dependent, and the result of decreased intracellular cAMP and increased PGE<sub>2</sub> levels.<sup>26,27</sup> In support of a role for the P2Y<sub>2</sub> receptor in modulating AVP-mediated changes in CD water transport, infusion of AVP (for 90 min) increases P2Y<sub>2</sub> mRNA and protein levels in the CD of hydrated rats.<sup>28</sup> Therefore, it has been proposed that activation of basolaterally located P2Y<sub>2</sub> receptors by extracellular nucleotides significantly affects AQP2 abundance, and thereby AVP-mediated water reabsorption, in the CD.<sup>29</sup> Indeed, P2Y<sub>2</sub> knockout mice exhibit higher levels of AQP2 compared with wild-type mice, despite similar AVP levels.<sup>30,31</sup>

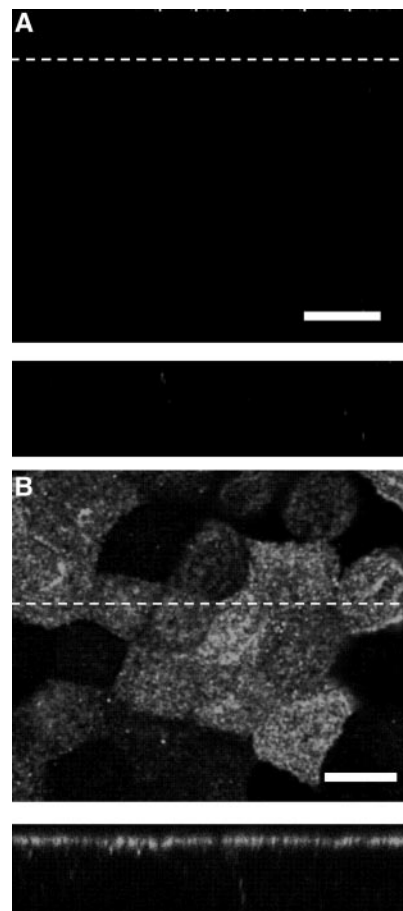
Activation of basolaterally localized P2Y<sub>2</sub> receptors has also been linked to the local control of amiloride-sensitive Na<sup>+</sup> reabsorption in CD PCs,<sup>22</sup> an effect that also occurs with luminal (apical) ATP. However, we have recently shown that the P2Y<sub>2</sub> subtype is not the only P2R to mediate a luminal effect of ATP on CD Na<sup>+</sup> transport.<sup>24</sup> Combining molecular and pharmacologic approaches, we found that activation of P2Y<sub>4</sub> (which is almost identical in its pharmacologic profile to P2Y<sub>2</sub>), P2X<sub>4</sub>, or P2X<sub>4/6</sub> receptors can also affect Na<sup>+</sup> transport. Furthermore, we showed that changing dietary Na<sup>+</sup> intake alters P2R mRNA and protein levels.<sup>24</sup>

Two questions arise from the previously published work: (1) Can AVP alter the protein abundance and mRNA levels of P2R other than the P2Y<sub>2</sub> subtype? (2) Can P2R other than the P2Y<sub>2</sub> 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,<sup>32,33</sup>



**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  $\mu$ m.

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 P2X<sub>1</sub>, P2X<sub>3</sub>, or P2X<sub>6</sub> subunit mRNA, or of P2Y<sub>6</sub> receptor mRNA (n = 6; Figure 2, A and B, respectively). In contrast, significant amounts of mRNA were detected for P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> subunits (Figure 2A), and for P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> receptors (Figure 2B); mRNA abundance had a rank order of P2Y<sub>2</sub> > P2X<sub>2</sub> = P2X<sub>4</sub> = P2Y<sub>1</sub> = P2Y<sub>4</sub> > P2X<sub>5</sub> = P2X<sub>7</sub>.

Cells treated with dDAVP showed a significant increase in abundance of P2Y<sub>2</sub> mRNA (by 7-fold; n = 6; P < 0.01), a significant decrease in abundance of P2Y<sub>1</sub> mRNA (by 2.5-fold; n = 6; P < 0.01), but no change in P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>7</sub>, or P2Y<sub>4</sub> mRNA levels (Figure 2, A and B). P2X<sub>1</sub> mRNA levels increased significantly in cells treated with dDAVP (Figure 2A). Rank order of abundance for P2R mRNA in cells treated with dDAVP was P2Y<sub>2</sub> > P2X<sub>1</sub> = P2X<sub>4</sub> > P2X<sub>2</sub> = P2Y<sub>1</sub> = P2Y<sub>4</sub> > P2X<sub>5</sub> = P2X<sub>7</sub>.

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**

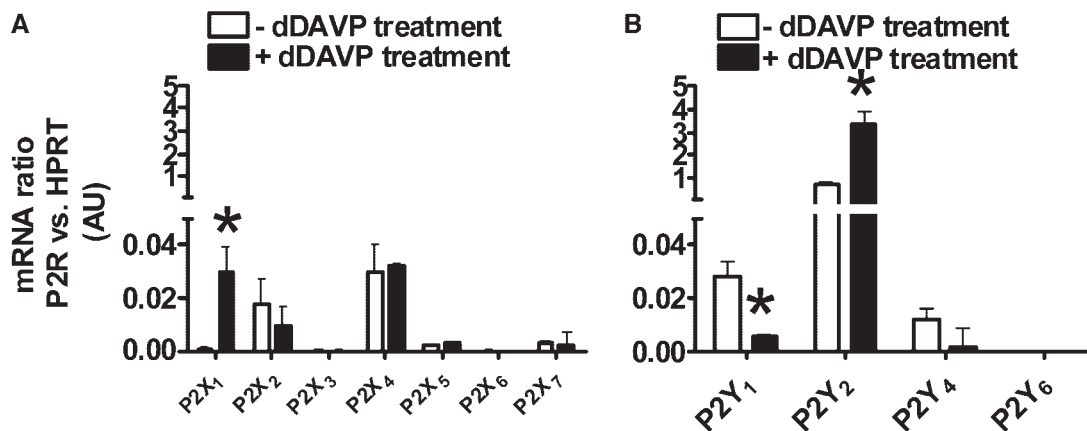
To investigate P2R protein localization in untreated mpkCCD cells, monolayers of cells (n = 6) were stained for different P2R using specific antibodies. Positive immunofluorescence for

P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors was seen apically, whereas P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> receptor subunits and P2Y<sub>2</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub> receptors were located throughout the cytoplasm, toward the cell borders and in the perinuclear region (Figure 3A). Immunofluorescent staining for P2X<sub>1</sub>, P2X<sub>3</sub>, and P2X<sub>6</sub> subunits and the P2Y<sub>6</sub> receptor was not seen. These data broadly agree with the real-time PCR findings.

To corroborate the apical localization of P2Y<sub>1</sub> and P2Y<sub>4</sub> 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 [V<sub>h</sub>] = -60 mV) cells in a confluent monolayer. Agonists were chosen according to their P2R selectivity (see Table 1).<sup>24,34</sup> The amplitudes of 10 μM agonist-evoked currents were 584 ± 59 pA (for ATP; n = 5), 540 ± 48 pA (for UTP; n = 5), 226 ± 26 pA (for ADP; n = 5), 29 ± 11 pA (for BzATP; n = 5), 343 ± 29 pA (for ATPγS; n = 5), and 234 ± 40 pA (for 2meSADP; n = 5); consistent with apical P2Y<sub>1</sub> and P2Y<sub>4</sub> receptor localization.

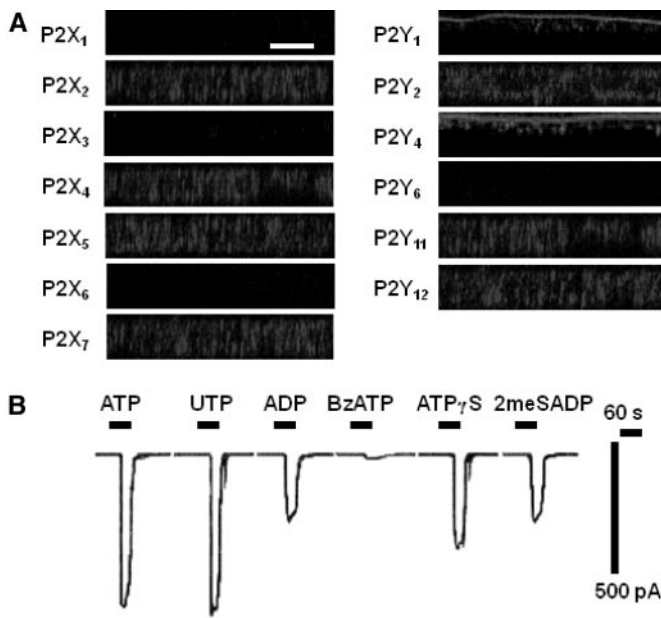
To test whether dDAVP alters the abundance and/or localization of P2R, mpkCCD cells were treated with dDAVP and subjected to immunocytochemistry using P2R-specific antibodies. Treatment with dDAVP (1 nM; 96 h; basolateral medium) resulted in positive immunostaining for P2X<sub>1</sub> and P2X<sub>2</sub> receptor subunits in the apical regions, and basolateral staining for P2Y<sub>2</sub> receptors (Figure 4, A and B), suggesting that dDAVP induces the synthesis of P2X<sub>1</sub>, and targets P2X<sub>1</sub>, and P2X<sub>2</sub> receptor subunits, as well as P2Y<sub>2</sub> 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-



**Figure 2.** Effect of dDAVP on P2R mRNA levels in mpkCCD cells. Real-time PCR was used to compare P2X and P2Y receptor mRNA levels in monolayers of mpkCCD cells grown to confluence on transwell permeable supports maintained in either the absence or presence of 1 nM dDAVP (applied to the basolateral medium) for 96 h. The data are presented as a ratio of the P2R gene of interest to the constitutively expressed housekeeping gene HPRT. (A) Following dDAVP treatment, mRNA levels for P2X<sub>1</sub> subunits were significantly increased (n = 6; P < 0.01). (B) Following dDAVP treatment, mRNA levels for P2Y<sub>1</sub> receptors were significantly decreased (n = 6; P < 0.01), and mRNA levels for P2Y<sub>2</sub> receptors were increased (n = 6; P < 0.01).

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**Figure 3.** P2R localization in mpkCCD cells. Confocal microscopy was used to determine the cellular distribution of P2R in monolayers of mpkCCD cells grown to confluence on transwell permeable supports (maintained for 96 h in the absence of dDAVP). Images shown are x-z planes. Monolayers were imaged in x-z planes from apical to basal boundaries of the cells. Scale bar represents 5  $\mu$ m. (A) Positive immunostaining (CY3) for P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors was seen in the apical region, whereas P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> receptor subunits, and P2Y<sub>2</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub> receptors, were located throughout the cytoplasm extending up to the cell border. (B) Typical trace showing a series of P2R agonist-evoked (10  $\mu$ M) inward currents from a whole-cell nystatin-permeabilized patch-clamped mpkCCD(c14) cell in a confluent monolayer (grown on a glass coverslip and maintained for 96 h in the absence of dDAVP). The cell was voltage-clamped at  $-60$  mV, and there was 5 min interval between agonist applications. P2R agonists ATP, UTP, ADP, ATP $\gamma$ S, and 2meSADP evoked inward currents consistent with apical P2Y<sub>1</sub> and P2Y<sub>4</sub> activation.

itor H-89 and subjected to immunocytochemistry using P2X<sub>1</sub>-, P2X<sub>2</sub>-, and P2Y<sub>2</sub>-specific antibodies. Treatment with H-89 (1  $\mu$ M; 30 min; basolateral medium) abolished dDAVP-dependent P2X<sub>1</sub> synthesis (and apical localization), dDAVP-dependent P2X<sub>2</sub> apical localization, and dDAVP-dependent P2Y<sub>2</sub> 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.

**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)

	ATP	UTP	ADP	BzATP	ATP $\gamma$ S	2meSADP
P2Y <sub>1</sub>	agonist	i.a.	agonist	i.a.	agonist	agonist
P2Y <sub>4</sub>	agonist	agonist	i.a.	antagonist	agonist (partial)	i.a.

Information taken from King and Townsend-Nicholson, 2003; Wildman et al, 2008.<sup>24,34</sup> i.a., inactive agonist at 10  $\mu$ M.

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

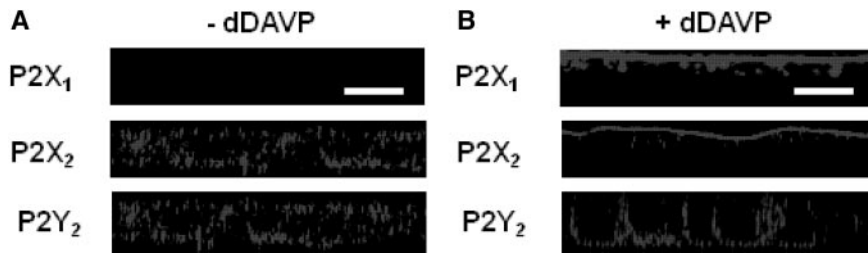
To investigate the effect of ATP on AQP2 localization, dDAVP-treated mpkCCD cell monolayers were treated with 1  $\mu$ M ATP $\gamma$ S (a stable analog of ATP with agonist activity at most P2R) for 2 h, added to either the apical or basolateral medium (n = 5). Immunostaining revealed that whereas AQP2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

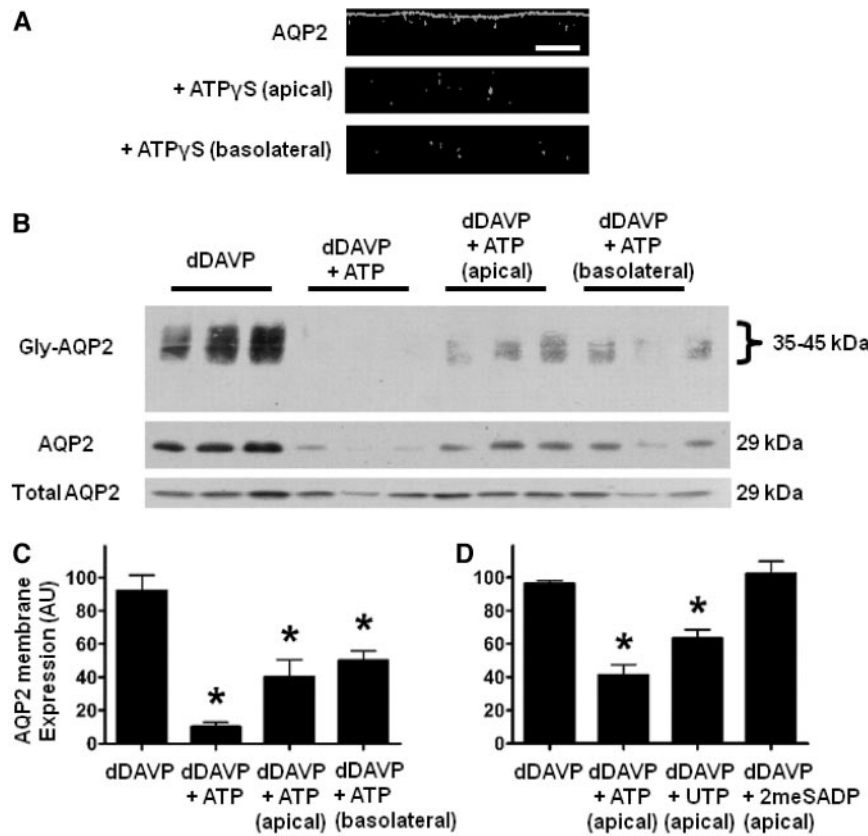
To investigate which of the apically localized P2R (P2X<sub>1</sub>, P2X<sub>2</sub>, P2Y<sub>1</sub> and/or P2Y<sub>4</sub>; see Figures 3, A and B, and 4) causes internalization of AQP2, a selection of partially selective P2R agonists was added to the apical medium of cultured mpkCCD cells for 2 h. Subsequent cell surface biotinylation, and semiquantification of AQP2 abundance by immunoblotting, showed that AQP2 was internalized from the plasma membrane when ATP (100  $\mu$ M; a potent agonist at P2X<sub>1</sub>, P2X<sub>2</sub>, and P2Y<sub>4</sub> receptor subtypes) or UTP (100  $\mu$ M; an agonist selectively favoring the P2Y<sub>4</sub> receptor subtype), but not 2meSADP (100  $\mu$ M; an agonist selective for the P2Y<sub>1</sub> receptor subtype), was added (Figure 5D). AQP2 membrane abundance was reduced to a greater extent by ATP than by UTP. These data indicate that apical P2Y<sub>4</sub> receptors and P2X<sub>1</sub> and/or P2X<sub>2</sub> receptors, but not P2Y<sub>1</sub>, 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  $\mu$ M) added to both sides (apical and basolateral) for 8 h. Subsequent immunoblotting for AQP2 and  $\beta$ -actin (to normalize for the amount of protein loaded) indicated that



**Figure 4.** Effect of dDAVP on localization of P2R in mpkCCD cells. Confocal microscopy was used to determine the cellular distribution of P2R in monolayers of mpkCCD cells grown to confluence on transwell permeable supports. Images shown are x-z planes. Monolayers were imaged in the x-z plane from apical to basal boundaries of the cells. Scale bar represents 5  $\mu$ m. (A) In cells untreated with dDAVP, positive immunostaining (Cy3) for P2X<sub>2</sub> receptor subunits and P2Y<sub>2</sub> receptors was found throughout the cytoplasm up to the cell border. (B) Treatment of cells with dDAVP (1 nM, applied to the basolateral medium for 96 h) resulted in positive immunostaining (Cy3) for P2X<sub>1</sub> and P2X<sub>2</sub> subunits in the apical domain, and for P2Y<sub>2</sub> receptors in the basolateral domain. Not shown, staining for P2X<sub>3</sub> and P2X<sub>6</sub> subunits, and P2Y<sub>6</sub> receptors, was still not seen in the presence of dDAVP; dDAVP treatment also did not alter the positive immunostaining for P2Y<sub>1</sub> or P2Y<sub>4</sub> receptors in the apical domain, or affect the pattern of immunostaining for P2X<sub>4</sub>, P2X<sub>5</sub> or P2X<sub>7</sub> subunits, or P2Y<sub>11</sub> or P2Y<sub>12</sub> receptors throughout the cytoplasm (as seen in Figure 3).

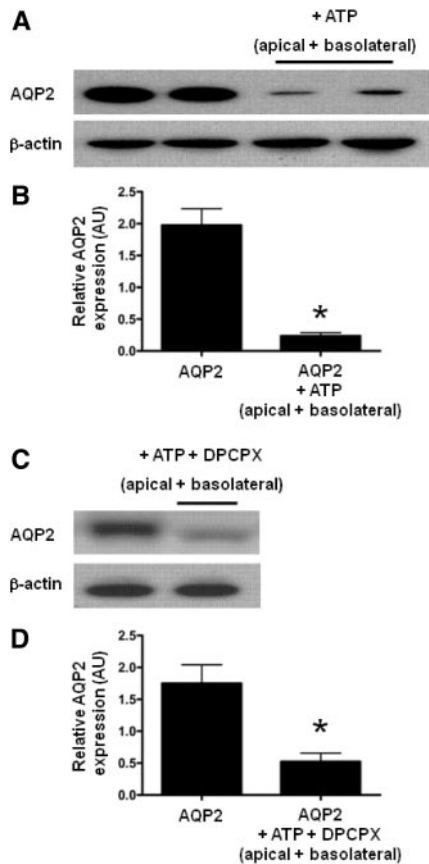


**Figure 5.** Effect of ATP on localization and membrane abundance of AQP2 in mpkCCD cells. (A) Confocal microscopy was used to determine the cellular distribution of AQP2 in monolayers of mpkCCD cells grown to confluence on transwell permeable supports following treatment with 1 nM dDAVP (applied to the basolateral medium) alone for 96 h, or along with 1  $\mu$ M ATP $\gamma$ S (applied to either the apical or basolateral medium) for the final 2 h. Monolayers were imaged in x-z planes. In cells where ATP $\gamma$ S was added to the apical or basolateral medium, positive immunostaining for AQP2 was no longer evident in the apical domain (see top panel), but instead was present weakly throughout the cytoplasm. Scale bar represents 5  $\mu$ m. (B) Confluent mpkCCD monolayers were stimulated with 1 nM dDAVP (96 h, basolateral medium) and subsequently treated with dDAVP only (dDAVP) or together with 100  $\mu$ M ATP on both the apical and basolateral sides (dDAVP+ATP), the apical side only (dDAVP+ATP [apical]), or the basolateral side only (dDAVP+ATP [basolateral]), for 2 h. Cells were then subjected to a cell surface biotinylation assay. Biotinylated proteins were pulled down and immunoblotted for AQP2 (Gly-AQP2 and AQP2). Total lysates were also immunoblotted for AQP2 (Total AQP2). (C) The signals from B were scanned and the amount of AQP2 was semiquantified in arbitrary units (AU). Significant differences from untreated cells are indicated by asterisks. The inhibitory effects of apical and basolateral ATP on AQP2 surface abundance were additive. (D) The signals from an experiment similar to that described in B, except that 100  $\mu$ M ATP, 100  $\mu$ M UTP or 100  $\mu$ M 2meSADP was present on the apical side for 2 h, were scanned and the amount of AQP2 was semiquantified in AU. Significant differences from untreated cells are indicated by asterisks. Biotinylation experiments were performed in triplicates in two independent experiments.

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  $\mu$ M) and DPCPX (10  $\mu$ M; a nonselective adenosine, P1, receptor antagonist) added to both

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 ATP $\gamma$ S (100  $\mu$ M; a stable analog of ATP with agonist activity at most P2R) added to both sides for 8 h; subsequent immunoblotting for AQP2 demonstrated that ATP $\gamma$ S reduced AQP2



**Figure 6.** Effect of extracellular ATP on AQP2 protein levels in mpkCCD cells. (A) Confluent monolayers of mpkCCD cells were stimulated with 1 nM dDAVP for 4 d, subsequently treated with dDAVP only or together with 100  $\mu$ M ATP for 8 h, and subjected to immunoblotting for AQP2. (B) The signals from A were scanned and the amount of AQP2 was semiquantified in AU. A significant difference in relative AQP2 abundance between untreated and treated cells is indicated by an asterisk. (C) As in A, except cells were left untreated or treated with 100  $\mu$ M ATP and 10  $\mu$ M DPCPX (an adenosine receptor antagonist) for 8 h. (D) The signals from C were scanned and the amount of AQP2 was semiquantified in AU. The asterisk indicates a significant difference. Immunoblotting experiments were performed in duplicates in two independent experiments.

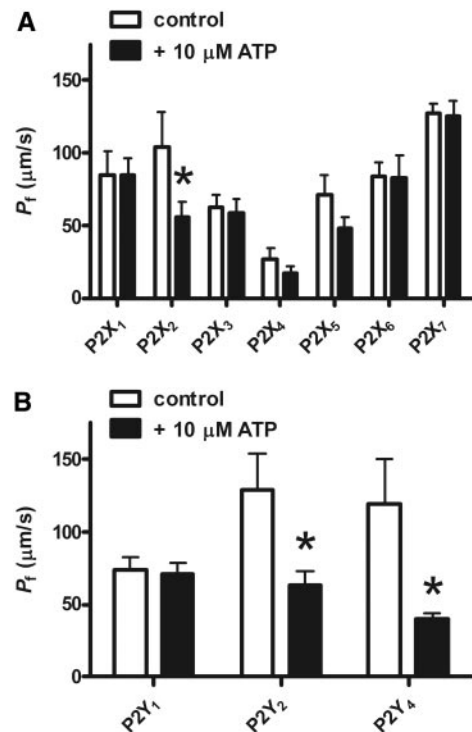
protein levels to a similar degree as that seen for ATP (data not shown). These findings suggest that adenosine is unlikely to be responsible for the reduction in AQP2 membrane abundance observed with ATP.

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

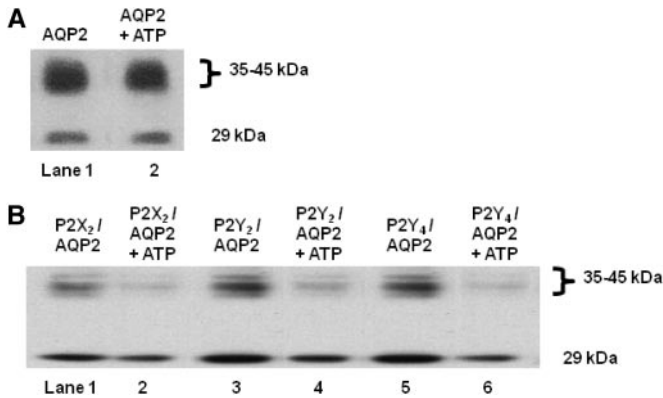
To help determine which of the P2R localized in mpkCCD cells could mediate the observed downregulation of AQP2 abundance, the P2R localized in the plasma membrane of mpkCCD cells with or without dDAVP (and a number of others) were co-expressed with AQP2 in *Xenopus* oocytes, and the oocytes subjected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

AQP2-mediated water permeability ( $P_f$ ) was significantly decreased following P2R activation in oocytes co-expressing P2X<sub>2</sub> (by 46  $\pm$  8%; n = 6;  $P$  < 0.01; Figure 7A), P2Y<sub>2</sub> (by 53  $\pm$  7%; n = 6;  $P$  < 0.01; Figure 7B) or P2Y<sub>4</sub> receptors (by 57  $\pm$  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<sub>1</sub>, 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<sub>1</sub>, P2X<sub>3</sub>, or P2X<sub>4</sub> 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<sub>2</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub>), plasma membrane abundance of



**Figure 7.** Effect of P2R activation on AQP2-mediated water permeability in *Xenopus* oocytes. Comparison of osmotic water permeability ( $P_f$ ) in *Xenopus* oocytes co-expressing AQP2 and the P2R of interest, in the absence or presence of 10  $\mu$ M ATP (15 min preincubation).  $P_f$  was determined from monitoring and measuring cell swelling (at 1.7 s intervals for 1 min) after cells were placed in a hypotonic solution. Significant differences from untreated cells are indicated by asterisks. (A) Activation of co-expressed P2X<sub>2</sub> receptors by extracellular ATP resulted in a significant decrease in  $P_f$  (n = 6;  $P$  < 0.01). (B) Activation of co-expressed P2Y<sub>2</sub> or P2Y<sub>4</sub> receptors also resulted in significant decreases in  $P_f$  (n = 6;  $P$  < 0.01). These data show that activation of P2X<sub>2</sub>, P2Y<sub>2</sub>, or P2Y<sub>4</sub> subtypes inhibits AQP2 activity within 15 min.



**Figure 8.** Activation of P2X<sub>2</sub>, P2Y<sub>2</sub>, or P2Y<sub>4</sub> receptors decreases plasma membrane abundance of AQP2 in *Xenopus* oocytes. Typical Western blot analysis of AQP2 protein in oocyte plasma membrane homogenates. (A) Oocytes expressing AQP2 in the absence (lane 1) or presence of 10 μM ATP (15 min; lane 2). The data show that extracellular ATP in the absence of P2R expression does not directly affect AQP2 membrane abundance. (B) Oocytes co-expressing AQP2 with P2X<sub>2</sub> in the absence (lane 1) or presence of 10 μM ATP (15 min; lane 2); co-expressing AQP2 with P2Y<sub>2</sub> in the absence (lane 3) or presence of 10 μM ATP (15 min; lane 4); and co-expressing AQP2 with P2Y<sub>4</sub> in the absence (lane 5) or presence of 10 μM ATP (15 min; lane 6). The data show that P2 receptor-mediated inhibition of cell swelling in *Xenopus* oocytes is associated with removal of AQP2 protein from the oocyte plasma membrane within 15 min.

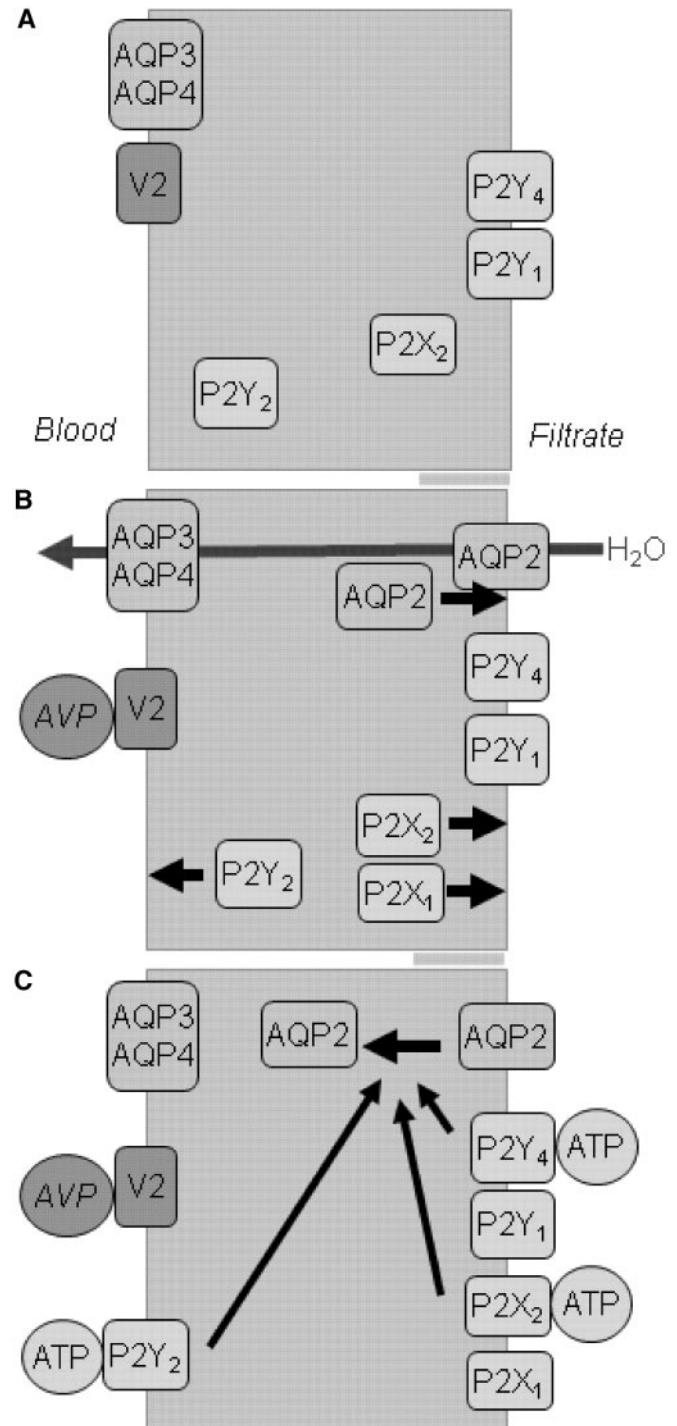
AQP2 was reduced. This is consistent with P2R-mediated inhibition of P<sub>f</sub> by P2X<sub>2</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> receptors by removal of AQP2 protein from the plasma membrane (n = 3; Figure 8B).

**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) P2Y<sub>1</sub> and P2Y<sub>4</sub> 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 P2X<sub>1</sub> recep-

**Figure 9.** Vasopressin alters P2R abundance and localization, and P2R activation results in the internalisation of AQP2. (A) Immunocytochemistry and patch clamp electrophysiology demonstrate that P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors are localized in the apical membrane of mpkCCD cells. (B) Treatment with dDAVP (acting via basolateral V2 receptors) results in the translocation of P2X<sub>2</sub> to the apical membrane, P2Y<sub>2</sub> to the basolateral membrane, and the synthesis and sorting (or trafficking) of P2X<sub>1</sub> and AQP2 to the apical membrane. (C) Addition of ATP to either the basal or apical side causes AQP2 to be internalized in mpkCCD cells. Experiments using a *Xenopus* oocyte swelling assay suggest that the effects of ATP are mediated via P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2X<sub>2</sub> activation.

tor; (3) AVP induces cAMP-dependent translocation and subsequent localization of P2X<sub>2</sub> and P2Y<sub>2</sub> receptors in the apical and basolateral membrane, respectively; (4) activation of basolaterally localized P2Y<sub>2</sub> receptors, and of apically localized P2X<sub>2</sub> and P2Y<sub>4</sub> 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.



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## P2R Abundance and Localization in mpkCCD Cells

As already mentioned, in cells untreated with AVP, we demonstrated localization of P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors in the apical membrane (corroborated by pharmacologic characterization); and of P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> subunits and P2Y<sub>2</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub> receptors throughout the cytoplasm. In cells treated with AVP, in which AQP2 is localized in the apical membrane, we demonstrated apical localization of P2X<sub>1</sub> and P2X<sub>2</sub> subunits, and of P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors, and basolateral localization of P2Y<sub>2</sub> receptors; while P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>7</sub> subunits and P2Y<sub>11</sub> and P2Y<sub>12</sub> receptors remained within the cytoplasm. Furthermore, using real-time PCR, we showed that AVP treatment increases mRNA levels of apically localized P2X<sub>1</sub> and basolaterally localized P2Y<sub>2</sub> receptors (Figure 2). Taken together, these data suggest that AVP induces not only AQP2 translocation and abundance, but also P2X<sub>1</sub> subunit and P2Y<sub>2</sub> receptor synthesis and membrane trafficking, and P2X<sub>2</sub> subunit membrane trafficking.

Our immunocytochemistry findings of P2R localization in mpkCCD cells agree broadly with previous reports of the presence of P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub> subunits and P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> receptors in rodent CD PCs.<sup>12,24,35–39</sup> Although others have not reported P2Y<sub>1</sub> receptor protein localization in the CD or in other PC-like cells in culture, P2Y<sub>1</sub> mRNA has been detected in rat CD.<sup>40</sup>

In contrast to our findings that chronic dDAVP treatment (96 h) causes an increase in P2Y<sub>2</sub> mRNA (Figure 2) and translocation of P2Y<sub>2</sub> receptors into the basolateral membrane (Figure 4), Sun and colleagues reported that chronic dDAVP infusion (120 h) in rats decreases P2Y<sub>2</sub> protein levels in the inner medullary CD.<sup>28</sup> The same group also reported that chronic water loading (48 h), which should lower endogenous AVP levels, increases P2Y<sub>2</sub> abundance.<sup>27</sup> In these *in vivo* studies, as acknowledged by the authors, the changes in P2Y<sub>2</sub> abundance could have been due to either a direct effect of AVP or changes in medullary tonicity. Given our findings, the latter seems a more likely explanation: an increase in tonicity causing suppression, and a decrease causing enhancement, of P2Y<sub>2</sub> abundance.

In addition to finding that chronic AVP treatment alters P2Y<sub>2</sub> abundance and localization in mpkCCD cells, we also found changes in the synthesis and apical localization of P2X<sub>1</sub> receptors, and the translocation and subsequent apical localization of P2X<sub>2</sub> receptors. We have shown recently that protein localization and mRNA levels of P2R in the rat CD can be affected by changes in dietary Na<sup>+</sup> intake.<sup>24</sup> We found that P2X<sub>1</sub>, P2X<sub>4</sub> and P2X<sub>6</sub> subunits, and P2Y<sub>4</sub> receptors, were up-regulated by a low Na<sup>+</sup> diet, an effect we attributed to increased circulating aldosterone levels. Activation of the apically localized P2Y<sub>4</sub> receptor inhibits the CD epithelial Na<sup>+</sup> channel (ENaC), whereas the apical P2X<sub>4</sub> subunit can inhibit or stimulate this channel. For the apical P2Y<sub>4</sub> receptor at least, this suggests the possibility of a coupling between its inhibitory effect on Na<sup>+</sup> and water transport in the CD. Interestingly, Schafer and Chen have reported that in Na<sup>+</sup>-depleted rats the

stimulatory effect of AVP on Na<sup>+</sup> and water transport in the CD is significantly reduced.<sup>41</sup>

## P2R-mediated Internalization of AQP2

Until recently, most attention in the CD has focused on P2Y<sub>2</sub> receptors, reinforced by recent studies of a P2Y<sub>2</sub> knockout mouse,<sup>31</sup> and it has already been established that activation of basolaterally localized P2Y<sub>2</sub> receptors by extracellular nucleotides can inhibit AQP2-mediated water transport in the CD.<sup>12–15</sup> This inhibition of AVP-stimulated, AQP2-mediated water transport is PLC- and PKC-dependent, and results in decreased intracellular cAMP and increased PGE<sub>2</sub>,<sup>12,13,15,42</sup> which is known to cause AQP2 internalization.<sup>43</sup>

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 P2Y<sub>2</sub> receptors in the terminal segment of the inner medullary CD is without significant effect on AQP2-mediated water reabsorption,<sup>15</sup> an effect by other apical P2R subtypes was not excluded in that study. Here we report that activation of apically localized P2X<sub>2</sub> and P2Y<sub>4</sub> receptors, rather than apical P2Y<sub>2</sub>, 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 P2Y<sub>4</sub>-mediated inhibition of AVP-sensitive water reabsorption is similar to that for P2Y<sub>2</sub> and is G<sub>q</sub> protein-coupled. However, from our recent study of apical P2R control of Na<sup>+</sup> transport, we know that P2X signal transduction involves stimulation of PI3K,<sup>24</sup> which when activated has also been shown to stimulate retrieval of AQP2 from the apical membrane.<sup>44</sup>

## 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.<sup>42</sup> 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 cilium.<sup>42,45</sup> 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 P2Y<sub>2</sub> 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 *in vitro* model, we have confirmed the location and function of the basolateral P2Y<sub>2</sub> receptor in mediating ATP inhibition of AVP-dependent water transport along the CD, but in addition, as with Na<sup>+</sup> transport, we have defined novel P2R-mediated inhibition of AVP-dependent water transport through apically located P2R. This apical regulation involves P2X<sub>2</sub> subunits and P2Y<sub>4</sub> (rather than P2Y<sub>2</sub>) 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 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml EGF, 5 μg/ml insulin, 20 mM D-glucose, 2% FCS, and 20 mM HEPES [pH 7.4]).<sup>33</sup> Cells were seeded at a density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> on semipermeable filters (Transwell<sup>®</sup>, 0.4 μm pore size, Corning Costar, Cambridge, USA); 1.13 cm<sup>2</sup> filters were used for immunocytochemistry or immunoblotting, and 4.7 cm<sup>2</sup> 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),<sup>32</sup> and the nucleotide compounds ATPγS, 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 ATPγS) 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 μg oligo(-dt) 12 to 18 primer and a first-strand cDNA synthesis kit (Superscript II RNase H<sup>-</sup> 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<sup>®</sup> 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.<sup>24</sup>

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 μg/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 μl 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 house-keeping 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.<sup>46</sup> Cells were incubated with affinity-purified rabbit anti-AQP2 antibodies or rabbit anti-P2R antibodies.<sup>24,47</sup> Noncommercial P2X antibodies were previously demonstrated to show subunit specificity.<sup>48</sup>

### Patch Clamp Electrophysiology

Patch clamp electrophysiology of cells grown on glass coverslips was performed as described previously.<sup>24</sup> The whole-cell configuration was used; this was achieved using a nystatin perforated-patch method. To establish the perforated-patch, nystatin (50 to 100 μg/ml) was added to the pipette solution (50 mM KCl, 90 mM K-gluconate, 3 mM MgCl<sub>2</sub>, 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 MΩ.

Cells were superfused (4 ml/min) with bathing solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> 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 ( $V_h$ ) 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 avoid run-down.

### Water Permeability (P<sub>f</sub>) Measurements

*Xenopus laevis* oocytes were obtained and defolliculated as described previously.<sup>49</sup> 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<sub>1</sub>, P2X<sub>3</sub>, P2X<sub>6</sub>, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub>, 50 ng; rat P2X<sub>2</sub> and P2X<sub>7</sub>, 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<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, 0.41 mM CaCl<sub>2</sub>, and 10 mM Hepes [pH 7.4]) and was supplemented with 50 μg/L gentamicin sulfate, then kept at 4 °C until used in experiments. Recombinant cRNA was not available for P2Y<sub>6</sub>, P2Y<sub>11</sub>, or P2Y<sub>12</sub> receptors.

Oocytes were analyzed after 48 h in a swelling assay as described previously.<sup>7</sup> Oocyte swelling was performed at 22 °C after transfer

from 200 to 70 mOsm/kg. Where stated, oocytes were preincubated with ATP (10  $\mu$ M) for 15 min before the swelling assay started.

### Immunoblotting

Immunoblotting and biotinylation experiments were performed as described previously.<sup>5,7</sup> Affinity-purified rabbit anti-AQP2 antibodies (1:3000)<sup>47</sup> or mouse anti- $\beta$ -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  $\pm$  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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