Counteracting vasopressin-mediated water reabsorption by ATP, dopamine, and phorbol esters: mechanisms of action

Michelle Boone,1 Marleen L. A. Kortenoeven,2 Joris H. Robben,2 Grazia Tamma,1 and Peter M. T. Deen2

1Department of General and Environmental Physiological, University of Bari, Bari, Italy; and 2Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted 5 May 2010; accepted in final form 30 December 2010

Boone M, Kortenoeven ML, Robben JH, Tamma G, Deen PM. Counteracting vasopressin-mediated water reabsorption by ATP, dopamine, and phorbol esters: mechanisms of action. Am J Physiol Renal Physiol 300: F761–F771, 2011. First published January 5, 2011; doi:10.1152/ajprenal.00247.2010.—Water homeostasis is regulated by a wide variety of hormones. When in need for water conservation, vasopressin, released from the brain, binds renal principal cells and initiates a signaling cascade resulting in the insertion of aquaporin-2 (AQP2) water channels in the apical membrane and water reabsorption. Conversely, hormones, including extracellular purines and dopamine, antagonize AVP-induced water permeability, but their mechanism of action is largely unknown, which was investigated here. Addition of these hormones to mpkCCD cells decreased total and plasma membrane abundance of AVP-induced AQP2, partly by increasing its internalization to vesicles and lysosomal degradation. This internalization was ubiquitin dependent, because the hormones increased AQP2 ubiquitination, and the plasma membrane localization of AQP2-K270R, which cannot be monoubiquitinated, was unaffected by these hormones. Both hormones also increased AQP2 phosphorylation at S261, which followed ubiquitination, but was not essential for hormone-induced AQP2 degradation. A similar process occurs in vivo, as incubation of dDAVP-treated kidney slices with both hormones also resulted in the internalization and S261 phosphorylation of AQP2. Both hormones also reduced cAMP and AQP2 mRNA levels, suggesting an additional effect on AQP2 gene transcription. Interestingly, phorbol esters only reduced AQP2 through the first pathway. Together, our results indicate that ATP and dopamine counteract AVP-induced water permeability by increasing AQP2 degradation in lysosomes, preceded by ubiquitin-dependent internalization, and by decreasing AQP2 gene transcription by reducing the AVP-induced cAMP levels.

water transport; mpkCCD cells; aquaporin-2 water channel

VASOPRESSIN (AVP)-regulated maintenance of water balance in the renal collecting duct occurs via alternating cell surface expression of the water channel aquaporin-2 (AQP2) and depends on the body’s need for water conservation. In states of hypernatremia and hypovolemia, vasopressin (AVP) is released into the bloodstream, interacts with its renal type 2 receptors, and initiates a signal transduction cascade (32). This includes activation of adenylate cyclase, a rise in intracellular cAMP and calcium levels, activation of PKA and its recruitment to AQP2-containing vesicles, and subsequent phosphorylation of AQP2 at Ser256 (10, 20, 35). This phosphorylation is essential for the redistribution of tetrameric AQP2 from intracellular storage vesicles to the apical plasma membrane (18). In addition, cAMP also increases AQP2 expression via phosphorylation of the cAMP-responsive element binding protein (CREB), which activates the AQP2 promoter (27, 43). Due to the increased plasma membrane expression of AQP2, water is able to pass the apical membrane passively along an osmotic gradient and enters the blood via AQP3 and AQP4 water channels, which are constitutively expressed in the basolateral membrane. Once water balance is restored, AVP levels drop and AQP2 is internalized via ubiquitination at Lys270 (19).

Besides AVP, several other hormones regulate water balance by antagonizing AVP-induced water transport (4), but the underlying mechanism is poorly understood. Extracellular purines, ATP and UTP, decrease AVP-induced water permeability, which is at least partially mediated via the P2Y2 receptor, located in the basolateral membrane of principal cells (6, 21, 38, 45), but may also involve P2X2 and P2Y4 receptors (42). Moreover, dopamine, carbachol, an acetylcholine analog, and endothelin-I inhibit AVP-induced water permeability (9, 11, 22, 23, 28, 37).

Intracellularly, the action of these hormones shows overlap, but also differs. ATP/UTP and dopamine appear to attenuate the AVP-triggered increase in intracellular cAMP, which is thought to be conferred by activation of a counteracting protein kinase C (ATP/UTP) (21), or coupling of the hormone receptor to the inhibitory G (Gi) protein (dopamine) (23). In contrast, carbachol reduced AVP-induced water transport via a PKC-dependent pathway but did not impair AVP-induced cAMP production (11, 26).

At present, however, it is unknown whether the reduced AVP-induced water permeability induced by these hormones involves AQP2 degradation and, if so, whether this occurs via AQP2 ubiquitination, internalization, and degradation, as we recently reported to occur with forskolin removal or addition of phorbol ester TPA, a drug that can activate PKC (19, 33). Moreover, it is unknown whether any of these hormones employs phosphorylation of AQP2 at any other site than Ser256, as it has recently been shown that AQP2 can also be phosphorylated at S261, S264, and S269 and that pS261-AQP2 is predominantly internalized with forskolin removal (21, 33). Therefore, to obtain more insight into the mechanism by which hormones counteract the action of AVP on AQP2, we here analyzed in detail the effect and underlying mechanism of ATP and dopamine on AVP-induced AQP2 abundance.

MATERIALS AND METHODS

Chemicals and reagents. [Deamino-Cys1, D-arg8]-vasopressin (dDAVP), ATP, UTP, dopamine, forskolin, cycloheximide, and chloroquine were purchased from Sigma (St. Louis, MO). dDAVP was administered to the basolateral side only. All other compounds were administered to both the apical and basolateral sides.
Ex vivo immunofluorescence. Female Sprague-Dawley rats were anesthetized with ether and euthanized by decapitation. The experiments performed were approved by the Ministry of Health (authorization no. 23/98-A), and animals were housed according to local and international requirements. Kidneys were quickly removed, and sections of ~0.5 mm were made and divided in four groups. The sections were equilibrated for 10 min in a buffer containing (in mM) 118 NaCl, 16 HEPES, 17 Na-HEPES, 14 glucose, 3.2 KCl, 1.5 CaCl₂, 1.8 MgSO₄, and 1.8 K$_2$PO₄ (pH 7.4). AQP2 trafficking was stimulated in the same buffer and at 37°C with 1 nM dDAVP for 15 min followed by a 30-min incubation with dDAVP alone or in combination with either 0.1 mM dopamine or ATP, or 100 nM TPA. Next, the kidney sections were fixed overnight in 4% paraformaldehyde at 4°C, infiltrated with 30% sucrose in PBS for 24 h, embedded in Cryomatrix (DDK, Milan, Italy) with dry ice, and cut with a cryostat to obtain 5-μm sections.

To stain for AQP2, the kidney sections were washed three times with PBS, blocked with 1% PBS-BSA for 1 h, incubated with our affinity-purified rabbit 1:1,000-diluted antibodies raised against the 20-amino acid N terminal of the polyphosphorylated region of human AQP2 (CLKGLEPLEPDTWEERVRQRQ; pre-C tail) for 2 h, and washed three times with PBS. After the washing, the sections were incubated with 1:1,000 diluted goat anti-rabbit antibodies coupled to Alexa 488 (Invitrogen, Milan, Italy), rinsed three times with PBS, blocked with 1% PBS-BSA for 1 h, incubated with affinity-purified rabbit anti-AQP2 antibodies (1:100) and goat anti rabbit antibodies coupled to Alexa 488 (1:100, Molecular Probes, Eugene, OR) with PBS, rinsed three times with PBS, and incubated with 1:1,000 diluted goat anti-rabbit antibodies coupled to Alexa 488 (Invitrogen, Milan, Italy), rinsed three times with PBS, and mounted in mounting medium containing 50% glycerol in 0.2 M Tris- HCl, pH 8.0, in the presence of 2.5% n-propyl gallate. Images were obtained with a Leica TCS SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Cell culture. MpkCCD cells (clone 14) (12) were grown in a modified defined 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 epidermal growth factor, 5 μg/ml insulin, 20 mM B-glucose, 2% fetal calf serum, and 20 mM HEPES (pH 7.4)].

Cells were seeded at a density of 1.5 × 10⁵ cells/cm² on semipermeable filters (Transwell, 0.4-μm pore size, Corning Costar, Cambridge, MA). For immunocytochemistry or immunoblotting, 1.13-cm² filters were used, and 20-cm² filters were used for biotinylation experiments. The cells remained in culture for 8 days before being analyzed. Cells were treated with 1 nM dDAVP for the last 4 days, to maximally induce AQP2 expression (24). Biotinylation was performed as described (5). The concentrations of the AVP-counteracting hormones ATP and dopamine were 100 μM, and the concentration of TPA used was 100 nM. Each experiment was done in triplicate and was repeated at least three times.

For the generation of cell lines stably expressing exogenous wild-type (wt) AQP2 of AQP2-K270R, the expression constructs pcB6-dBamHI-AQP2 and pcB6-dBamHI-AQP2-K270R (19), which encode the calcium phosphate method as previously described for Madin-Darby canine kidney (MDCK) cells (5). MDCK-wtAQP2 have been described (5).

For the generation of cell lines stably expressing exogenous wild-type (wt) AQP2 of AQP2-K270R, the expression constructs pcB6-dBamHI-AQP2 and pcB6-dBamHI-AQP2-K270R (19), which encode the calcium phosphate method as previously described for Madin-Darby canine kidney (MDCK) cells (5). MDCK-wtAQP2 have been described (5).

Immunoprecipitation. Twenty microliters of protein A agarose beads (Kem-En-Tec, Copenhagen, Denmark) per sample were washed three times in lysis buffer [1% Triton X-100, 150 mM NaCl, 25 mM HEPES (pH 7.4)]. Per sample, 1 μl of rabbit anti-AQP2 or guinea pig anti-AQP2 antibodies was added to 1 ml lysis buffer with protease inhibitors (1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml a-proclin) and rotated for 4 h at room temperature. Before use, the antibody-coupled beads were washed three times with lysis buffer. Cells were treated as described and lysed in 1 ml lysis buffer containing protease inhibitors and 20 mM N-ethylmaleimide (NEM). The samples were centrifuged at 12,000 g for 10 min, and the supernatant was incubated for 16 h with the antibody-coupled beads at 4°C. The beads were washed four times with lysis buffer, carefully dried, and resuspended in 30 μl of 1× Laemmli buffer with 0.1 M DTT. Subsequently, the samples were analyzed by immunoblotting for ubiquitin, AQP2-phospho-S261, or total AQP2 as indicated.

Statistical analyses. Films were scanned using a GS-690 Imaging Densitometer (Bio-Rad, Hercules, CA) and analyzed using Bio-Rad software. Statistical comparisons were made using one-way ANOVA.
and Bonferroni’s post hoc correction. $P < 0.05$ was considered significant.

RESULTS

Effect of ATP and dopamine on AQP2 abundance. To study the role of hormones counteracting AVP in vitro, we used the mouse cortical collecting duct (mpkCCD) cells as a model, because these cells show an AVP-induced expression of endogenous AQP2 (12). To determine whether AVP-counteracting hormones counteract AVP, mpkCCD cells were grown to confluence for 8 days, incubated for the last 4 days with dDAVP to generate a steady-state endogenous AQP2 abundance (24), and then treated for the last 2 or 8 h with the 100 μM ATP or dopamine in the continued presence of dDAVP. TPA,

![Figure 1](http://ajprenal.physiology.org/)

Fig. 1. Effect of TPA, ATP, and dopamine on [Deamino-Cys$^1$, c-arg$^8$]-vasopressin (dDAVP)-induced aquaporin-2 (AQP2) abundance. MpkCCD cells were grown for 4 days, incubated with 1 nM dDAVP for an additional 4 days, and treated with 100 nM TPA, 100 μM ATP, or 100 μM dopamine in the presence of dDAVP for the last 2 (A) or 8 h (B), or with UTP for 2 h (C). Subsequently, the cells were lysed and immunoblotted for AQP2. The signals were semiquantified using densitometry. One of three independent experiments is shown. The quantification shown here is pooled data from all 3 experiments. *Samples significantly ($P < 0.05$) different from controls.
Effect of ATP and dopamine on AQP2 internalization. MpkCCD cells were grown and treated as described in the legend of Fig. 1, except that the treatment with TPA, ATP, and dopamine was for 2 h. Subsequently, cells were subjected to cell surface biotinylation and lysed. Biotylated proteins were pulled down from the remaining solution and immunoblotted for AQP2 (plasma membrane). In addition, total lysates were immunoblotted for AQP2 (Total). The signals were semiquantified using densitometry. Triplicate samples and independent experiments were performed in triplicate, of which a representative experiment is shown. Graphs show pooled data from 3 experiments. *Samples significantly ($P < 0.05$) different from controls.

which is known to decrease AQP2 abundance in mpkCCD cells (19), was taken along as a control. Subsequent immunoblotting showed that both hormones and TPA decreased AQP2 abundance at 2 and 8 h hormone treatment (Fig. 1, A and B). Similarly, treatment with 100 μM UTP for 2 h also significantly reduced AQP2 abundance (Fig. 1C). These data indicated that mpkCCD cells can be used as a model to study the mechanism by which ATP and dopamine counteract AVP. As incubation for 8 h gave more consistent results throughout the various experiments, 8-h incubation was used for following experiments. Similarly, although lower concentrations of ATP and dopamine also affected AQP2 abundance, the above-mentioned concentrations of AVP-counteracting hormones gave more consistent results and were therefore selected for the experiments below.

Effect of ATP and dopamine on AQP2 internalization. The observed reduction in AVP-induced AQP2 abundance in the 8-h time frame can be due to increased AQP2 internalization and degradation, which is a relatively fast process, and/or reduced AQP2 transcription, which usually is a slow process. To determine whether these hormones employ AQP2 internalization, dDAVP-induced mpkCCD cells were left untreated or treated with TPA, ATP, or dopamine for 2 h and subjected to apical cell surface biotinylation assays. Immunoblotting of the biotinylated proteins and normalization for the respective total amounts of AQP2 revealed a significantly reduced AQP2 abundance in the apical membrane with TPA, ATP, and dopamine (Fig. 2). As anticipated for the short incubation time, none of the treatments significantly affected the total amount of AQP2.

Effect of ATP and dopamine on AQP2 ubiquitination. Recently, we showed that TPA induces short-chain ubiquitination of AQP2 in vitro and in vivo and that this ubiquitination precedes, and is essential for, AQP2 internalization and degradation upon TPA treatment in MDCK-AQP2 cells (19). As ubiquitination is more readily detectable in MDCK then in mpkCCD cells, we initially reverted to MDCK-AQP2 (3) cells for this purpose. Following forskolin treatment for 45 min, these MDCK-AQP2 cells were left untreated (control) or incubated with TPA, ATP, or dopamine for 15 min (optimal period for detecting ubiquitination in these cells), lysed, and subjected to AQP2 immunoprecipitation. Subsequent immunoblotting for ubiquitin revealed the typical strong 43- and 50-kDa and the weaker 58-kDa ubiquitinated AQP2 bands in all lanes, which were clearly increased for cells coincubated with ATP and dopamine and, to a higher extent, with TPA, compared with control cells (Fig. 3). Detection of immunoprecipitated equivalents with AQP2 antibodies (Fig. 3, bottom) revealed that similar amounts of AQP2 were loaded.

To assess the involvement of ubiquitination in the ATP- and dopamine-induced internalization of AQP2 from the plasma membrane in mpkCCD cells, these cells were stably transfected for wtAQP2 or the mutant AQP2-K270R, which cannot be short chain ubiquitinated (19). As shown in Fig. 4, treatment with forskolin alone results in apical plasma membrane localization of wtAQP2 and AQP2-K270R. In line with the ubiquitination data above, cotreatment with TPA, ATP, or dopamine induced internalization of wtAQP2 into vesicles. In contrast, the apical localization of AQP2-K270R remained unaffected by any of these compounds, demonstrating that ubiquitination of K270 is essential for hormone-induced internalization of AQP2 in mpkCCD cells.

Effect of ATP and dopamine on S261 phosphorylation. Besides phosphorylation of AQP2 at S256 by PKA, three additional

Fig. 2. Effect of TPA, ATP, and dopamine on AQP2 internalization. MpkCCD cells were grown and treated as described in the legend of Fig. 1, except that the treatment with TPA, ATP, and dopamine was for 2 h. Subsequently, cells were subjected to cell surface biotinylation and lysed. Biotylated proteins were pulled down from the remaining solution and immunoblotted for AQP2 (plasma membrane). In addition, total lysates were immunoblotted for AQP2 (Total). The signals were semiquantified using densitometry. Triplicate samples and independent experiments were performed in triplicate, of which a representative experiment is shown. Graphs show pooled data from 3 experiments. *Samples significantly ($P < 0.05$) different from controls.

Fig. 3. Effect of TPA, ATP, and dopamine on AQP2 ubiquitination. Confluent monolayers of Madin-Darby canine kidney (MDCK)-AQP2 cells were incubated with 5 × 10⁻⁵ M forskolin followed by coincubation with 100 nM TPA, 100 μM ATP, or 100 μM dopamine for the last 15 min. Cells were lysed and subjected to immunoprecipitation using rabbit anti-AQP2 antibodies. Samples were immunoblotted, and blots were incubated with mouse anti-ubiquitin (top) or guinea pig anti-AQP2 (bottom) antibodies. Protein masses (in kDa) are indicated on the left.
phosphorylation sites were identified in the C-terminal tail of AQP2, S261, S264, and S269 (15, 16). Interestingly, whereas AVP treatment increased phosphorylation at S256, AQP2-pS261 mainly localized in vesicles and AQP2 phosphorylation at S261 was decreased upon AVP incubation (15). To investigate whether the AVP-counteracting hormones affect the phosphorylation state of AQP2 at S261 and how this relates to ubiquitination of AQP2, mpkCCD-AQP2 and AQP2-K270R cells were left untreated or preincubated with 100 nM dDAVP, followed by coincubation with TPA, ATP, or dopamine for 15 min. Consistent with in vivo data, dDAVP reduced the pS261 29-kDa signals for wtAQP2 (Fig. 5A, top left). Upon coincubation of mpkCCD-AQP2 cells with TPA, ATP, or dopamine two forms of data were obtained. Often, TPA, ATP and dopamine did not change the signal for AQP2-pS261 at 29 kDa, but induced the appearance of a signal at 43 kDa (Fig. 5A, top left), a band that also appeared in the blot representing total AQP2 (Fig. 5A, bottom left). In other experiments, AQP2-pS261 of 29 kDa was also observed (not shown). For AQP2-K270R, the basal level of AQP2-pS261 also decreased upon treatment with dDAVP (Fig. 5A, top right). Cotreatment with TPA, ATP, and dopamine neither led to an increase in the 29-kDa signal, but also did not result in the appearance of the 43-kDa signal, as observed for wtAQP2. Total levels of AQP2 protein remained unaltered during the experiment (Fig. 5A, bottom). These data suggest that ubiquitination precedes pS261 in AQP2.

To examine this in more detail, we analyzed phosphorylation of S261 in wtAQP2 and AQP2-K270R expressing mpkCCD cells time dependent, after treatment with ATP and dopamine. As shown for ATP (Fig. 5B), wtAQP2 starts to get phosphorylated at S261 ~15 min after the start of ATP treatment. At this time point, however, it is predominantly the ubiquitinated form of AQP2 that is phosphorylated. At 30 min after ATP stimulation, when the extent of AQP2 ubiquitination has dropped (19), the signal for ubiquitinated AQP2-pS261 decreases, while that of 29-kDa AQP2-pS261 increased to a level that is sustained at later time points. In contrast, AQP2-K270R does not get phosphorylated at pS261 at any time point measured (Fig. 5B). Similar data were obtained for dopamine (not shown). Also, following treatment with ATP or dopamine at a more detailed early time frame, immunoblotting for ubiquitin revealed an increase in ubiquitinated AQP2 after 5 min, with a peak after 10–15 min (Fig. 5C, top), while pS261 signals of 29- and 43-kDa AQP2 mainly increased between 10 and 20 min after hormone addition (Fig. 5C, top middle). [Note that the 25-kDa band in the blot for ubiquitination is a background band, because it is present in mpkCCD cells not pretreated with dDAVP (see also input, bottom)]. The total

---

**Fig. 4.** Effect of TPA, ATP, and dopamine on the subcellular localization of AQP2 and its ubiquitination mutant AQP2-K270R. MpkCCD-wild-type (wt) AQP2 and AQP2-K270R cells were grown for 8 days, incubated with 5 × 10⁻³ M forskolin for 45 min to induce apical localization of AQP2, and were then left untreated (control), or treated with dDAVP in the presence of 100 nM TPA, 100 µM ATP, or 100 µM dopamine for 30 min. Subsequently, cells were fixed and subjected to immunocytochemistry for AQP2. Top view (XY) confocal images and their corresponding cross sections (XZ) are shown.

---

**AJP-Renal Physiol • VOL 300 • MARCH 2011 • www.ajprenal.org**
levels of immunoprecipitated and total AQP2 remained essentially unaltered (Fig. 5C, bottom). Together, these data indicated that hormone-induced phosphorylation of S261 indeed follows ubiquitination of AQP2.

In addition, we investigated the effects of intracellular calcium levels on ubiquitination and subsequent S261 phosphorylation of AQP2. However, as shown in Supplementary Fig. S1, exclusion of calcium by coincubation with BAPTA-AM did not affect ATP, dopamine, or TPA-induced ubiquitination or pS261 of AQP2 (all supplementary material for this article is available on the journal web site). These data indicated that these processes are calcium independent.
To test whether S261 phosphorylation is important in the hormone-induced degradation, mpkCCD cells stably expressing AQP2-S261A, which cannot be phosphorylated, or AQP2-S261D, which mimics its constitutively phosphorylated form, were treated with the hormones as above. Immunocytochemistry and immunoblotting (Supplementary Fig. S2), however, revealed that ATP, dopamine, and TPA induced internalization and degradation of AQP2-S261A and AQP2-S261D, similar to what was observed for wtAQP2. These data reveal that ubiquitination, but not the phosphorylation status of S261, is critical in the process of AQP2 internalization and its subsequent degradation.

**Effect of ATP and dopamine on AQP2 degradation.** In mpkCCD cells, TPA-induced internalization of AQP2 leads to its degradation via the lysosomal pathway, which can be inhibited by chloroquine (19, 39). To test whether the hormones also affect AQP2 degradation, dDAVP-pretreated cells were coincubated for 8 h with ATP, dopamine, or TPA in the presence or absence of cycloheximide to block protein synthesis, and in the presence or absence of chloroquine. Subsequent immunoblotting showed a significant reduction in AQP2 abundance when cells were incubated with these compounds (Fig. 6). This reduction was significantly prevented by blocking lysosomal protein degradation with chloroquine. Subsequent immunoblotting showed a significant reduction in AQP2 abundance when cells were incubated with these compounds (Fig. 6). This reduction was significantly prevented by blocking lysosomal protein degradation with chloroquine. Together, the data above reveal that ATP, dopamine, and TPA counteract AVP-induced water permeability by increasing the internalization of AQP2 from the plasma membrane in an ubiquitin-dependent manner to induce its lysosomal degradation.

**Effect of ATP and dopamine on AQP2 mRNA abundance.** Besides increased AQP2 internalization and degradation as shown above, the observed reduction in AVP-induced AQP2 abundance can partially be due to reduced AQP2 production. To determine whether the above-mentioned hormones affect AQP2 mRNA levels, cells were treated as above and subjected to quantitative RT-PCR. Using equal amounts of starting cDNA and following normalization for 18S mRNA, ATP gave a nearly 60% reduction in AQP2 mRNA, while dopamine showed a 30% reduction (Fig. 7). TPA did not reduce AQP2 mRNA levels.

**Effect of ATP and dopamine on cAMP production.** Binding of AVP to its V2 receptor induces an increase in cAMP, which results via activation of CREB in increased AQP2 gene transcription (27, 43). Considering the observed reduction in AQP2 mRNA levels with ATP and dopamine (Fig. 7), we tested whether the AVP-counteracting hormones used in the study reduce the steady-state cAMP levels induced by dDAVP. For this, mpkCCD cells were grown and pretreated with dDAVP as above, incubated with the phosphodiesterase inhibitor IBMX for 10 min, followed by treatment with ATP, dopamine, and TPA for 15 min in the continued presence of dDAVP and IBMX. Subsequent analysis of the cAMP levels revealed that ATP and dopamine significantly decreased cAMP levels, which was not observed with TPA (Fig. 8).

**Effect of ATP and dopamine ex vivo.** To investigate whether TPA, ATP, and dopamine also induce AQP2 internalization in kidney principal cells and whether this also coincides with increased phosphorylation of AQP2 at S261, rat kidneys slices were prepared and treated with the respective hormones. Immunoblotting revealed a significant increase in the phosphorylation of AQP2 at S261 in the presence of ATP and dopamine, but not TPA, compared to untreated controls (Fig. 9).

---

**Fig. 6.** Effect of TPA, ATP, and dopamine on lysosomal degradation of AQP2. MpkCCD cells were grown to confluence, treated with 1 nM dDAVP for 4 days, and then left untreated or incubated with 100 μM ATP or 100 μM dopamine with or without 100 μM chloroquine, dDAVP, and 50 μM cycloheximide for 8 h. Subsequently, cells were lysed and immunoblotted for AQP2 and signals quantified using densitometry. Quantified data represents pooled data from triplicate samples of 3 independent experiments. *Samples significantly (P < 0.05) different from the dDAVP alone control.

**Fig. 7.** Effect of ATP, dopamine, and TPA on AQP2 mRNA amount. MpkCCD cells were grown and treated as described in the legend of Fig. 6, except that cycloheximide was omitted. Cells were lysed, mRNA was isolated, and reverse transcribed to cDNA using random primers. Equal amounts of cDNA were subsequently subjected to Q-PCR using primers for mouse AQP2 and 18S (internal control). Following normalization against the 18S signal, the AQP2 mRNA amounts are expressed relative to control values.

---
and cAMP levels were determined with a cAMP enzyme immunoassay kit. Another 15 min in the presence of dDAVP and IBMX. Then, cells were lysed and cAMP levels were determined with a cAMP enzyme immunoassay kit.

Discussion

ATP and dopamine counteract AVP action by inducing ubiquitin-dependent AQ2 internalization and lysosomal degradation. Several studies have demonstrated a role for extracellular purines ATP and UTP and phenethylamine dopamine in the inhibition of AVP-induced water resorption (6–8, 21, 23, 37). Our present study reveals that the mechanism of action by which these hormones counteract vasopressin consists of two parts.

The first part involves the short-term ubiquitin-dependent internalization and lysosomal degradation of AQ2. Using mpkCCD cells stimulated with AVP to induce total and plasma membrane expression of endogenous AQ2, ATP and dopamine induced internalization of AQ2 from the apical membrane (Fig. 2) followed by lysosomal degradation (Fig. 6). Importantly, using kidney slices we could show that these short-term effects on AQ2 internalization mimicked the in vivo effects of ATP and dopamine on AQ2 localization (Fig. 9A). As we have shown for TPA-induced internalization of AQ2 (19), our data reveal that this internalization of AQ2 by ATP and dopamine is dependent on the ability to ubiquitinate AQ2 (Fig. 4). Using MDCK-AQP2 cells, we found that both hormones increase AQ2 ubiquitination, resulting in two main bands of 43 and 50 kDa, a weaker higher band of ~58 kDa and some smear (Fig. 3). This smear may represent glycosylated, K63-ubiquitinated AQ2 or possibly K29-polyubiquitinated AQ2. Polyubiquitination via K29 in ubiquitin is thought to be a general mechanism to degrade membrane proteins via the endoplasmic reticulum-associated degradation (ERAD) pathway. These different ubiquitination bands were not observed in precipitates of mock-transfected MDCK cells and are consistent with the addition of two to four ubiquitin moieties onto AQ2 (19). Possibly because of increased expression of deubiquitinating enzymes in dDAVP-induced mpkCCD cells, ubiquitinated AQ2 is not readily detectable in mpkCCD cells (see also below). However, our finding that wtAQ2, but not the constitutively deubiquitinated AQ2-K270R (19), is internalized in mpkCCD cells with both hormones (Fig. 4), indicates that ubiquitination is an important regulatory mechanism in mpkCCD cells as well and that ubiquitination of AQ2 is needed for both hormones to internalize AQ2.

The second part involves the long-term effects on AQ2 mRNA expression. As shown in Fig. 8 and consistent with in vivo data using perfused inner medullary collecting ducts (21, 26), ATP and dopamine also significantly reduce steady-state cAMP levels. As AQ2 gene expression is increased when CREB binds to the CRE present in the AQ2 promoter (27, 43), the reduced AQ2 mRNA levels observed with both hormones (Fig. 7) are likely a consequence of the reduced cAMP levels.

The reduction in cAMP with dopamine seems rather low, but it has to be taken into account that it is considered and even shown for AQ2 that intracellular cAMP levels are locally regulated, in which the kinase-anchoring proteins (AKAPs) play an important role (1, 34). AKAPs bind PKA, PKA substrates, phosphatases, and PDEs and target these proteins and thus cAMP-induced signaling cascades to various subcellular compartments to their unique targeting domains (13, 35). It will be interesting to test whether ATP and dopamine do decrease local pools of intracellular cAMP.

Short-chain ubiquitination of AQ2 precedes phosphorylation at S261. While the essentiality of S265 phosphorylation for AQ2 translocation to the apical membrane is unchallenged, several data reveal that internalization of AQ2 does not necessarily require S265 dephosphorylation, as activation of PKC by TPA (40), and also dopamine- and PGE2-induced internalization of AQ2 to intracellular vesicles occurred without reducing S265 phosphorylation (29, 44). Recently, we have shown that TPA-induced AQ2 endocytosis involves its short-chain ubiquitination at K270 (19), but it is unclear whether any of the three newly identified additional phosphorylation sites in the C-terminal tail of AQ2 may be involved, being S261, S264, and S269 (16). Interestingly, Hoffert et al. (15) showed that S261 phosphorylation of inner medullary collecting duct AQ2 was decreased upon AVP incubation (15). Here, we demonstrated that, in line with in vivo, dDAVP decreases phosphorylation of AQ2 at S261 (Fig. 5). More importantly, however, our data also reveal that internalization of AQ2 with ATP and dopamine coincides with an increase in S261 phosphorylation (Fig. 5B).

In mpkCCD cells stably transfected with AQ2, ATP, and dopamine treatment initially results in an increased pS261 signal of a 43-kDa band, while the 29-kDa band was not or only weakly increased (Fig. 5A). In two different time series, it appeared that 43-kDa AQ2-pS261 preceded AQ2-pS261 of 29 kDa (Fig. 5, B and C). This pS261-labeled 43-kDa band likely represents AQ2 bound by two ubiquitin moieties, because its mass is consistent with this (19), and the band is not observed in ATP- or dopamine-treated mpkCCD cells expressing AQ2-K270R (Fig. 5, A and B). Although we cannot exclude the possibility that our pS261 antibodies have a higher affinity for ubiquitinated than nonubiquitinated AQ2 and/or that ubiquitinated AQ2 is more prone to S261 phosphorylation than 29-kDa AQ2, these data indicate that AQ2 ubiq-
uitination precedes pS261 phosphorylation as an early event in the ATP/dopamine-induced AVP-counteracting pathway of AQP2 proteins from the plasma membrane to lysosomes. This phosphorylation of S261, however, was not essential for ATP/dopamine/TPA-induced internalization or degradation, because internalization and degradation of AQP2 proteins mimicking constitutively phosphorylated or dephosphorylated S261 (Supplemental Fig. S2) were similar to that of wtAQP2. Although these data may suggest that ubiquitination is needed before pS261 can occur, it is more likely that ubiquitination is a faster process or that pS261 of AQP2 occurs at an intracellular location (i.e., after ubiquitin-mediated endocytosis), because AQP2 that cannot be ubiquitinated (AQP2-K270R) is phosphorylated at S261 in unstimulated mpkCCD cells. The role of S261 phosphorylation in AQP2 thus remains to be established. Moreover, it is noteworthy that the localization of AQP2-S261A/D in mpkCCD cells with or without treatment of dDAVP is similar to that found by others in different cell types (15, 25), but is different from the localization we observed in MDCK cells (unpublished observations), which is likely due to expression of a different set of proteins in our MDCK type I cell model. It will be interesting to identify the protein(s) causing this difference in handling AQP2-S261A/D.

Recently, the first steps were taken to identify the kinases involved in the phosphorylation of AQP2 at S261. Although PKA is responsible for the phosphorylation of AQP2 at S256, Hoffert et al. (14) demonstrated that PKA is unable to phosphorylate S261. In vitro experiments where synthetic COOH-terminal AQP2 peptides were incubated with various purified MAP kinases demonstrated that JNK, p38, and CDK5/9 could potentially be involved in the phosphorylation of AQP2 at S261 (31). Although JNK, p38, and CDK5/9 are putative candidates, the identity of kinases
involved in the ATP- and dopamine-dependent S261 phosphorylation of AQP2 remains to be established.

**ATP and dopamine receptors are involved in AQP2 degradation.** Hormones can inhibit AVP-stimulated water permeability by decreasing cAMP levels, but can also exert their effects without affecting cAMP production, depending on the receptor subtype and subsequent G protein that is activated. In case the receptor couples to Gi, cAMP production will be inhibited, whereas upon Gq coupling, phospholipase C is activated and intracellular Ca2+ levels will increase. In AVP-stimulated mpkCCD cells, ATP and dopamine both decreased cAMP production (Fig. 8), suggesting that ATP and dopamine activate receptors that couple to Gi.

Purinergic receptors expressed in the collecting duct include P2Y1, P2Y2, and P2Y4 receptors. Our recent paper suggests the involvement of P2Y4 and P2X2 receptors, in addition to P2Y2, in AQP2-mediated water permeability in mpkCCD cells (42). Here, treatment with the more stable UTP yielded the same results as with ATP (Fig. 1C), which supports involvement of P2Y2 or P2Y4 in the ATP effect on AQP2, as UTP is more selective for these receptors compared with P2Y1, and has never been reported to bind to P2X receptors (17, 30). Alternatively, the effect of ATP may be mediated indirectly through EP receptors. Extracellular purines have been shown to stimulate P2Y2 receptor-mediated release of prostaglandin E2 and arachidonic acid (41), which activate the Gi coupled EP receptors EP1 and EP3.

Of the dopamine receptors, D2, D3, and D4 couple to Gi. Of these, the D4 receptors may be most likely to mediate the effect of dopamine on AQP2, since these receptors are expressed in the renal collecting duct (23, 37).

Considering the fact that TPA, which is thought to activate PKCs, does counteract vasopressin action through the short-term mechanism (AQP2 internalization, ubiquitination, lysosomal degradation) (Figs. 2, 3, and 6), but does, in contrast to ATP and dopamine, not affect reduce cAMP levels (Fig. 8), may indicate that ATP and dopamine indeed use a bimodal mechanism (direct PKC activation through Gq and indirect reduction of cAMP through activation of EP1/3 and Gi) to counteract vasopressin action. The identity of the purinergic and dopamine receptors involved and the precise mechanism by which activation of these receptors result in reduced cAMP and AQP2 levels remains to be established.

In summary, we demonstrated that ATP and dopamine counteract AVP-induced water permeability by two mechanisms, AQP2 internalization and lysosomal degradation, in an ubiquitin-dependent and pS261-related manner, and through decreased cAMP levels.

**ACKNOWLEDGMENTS**

Dr. M. A. Knepper, Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, Bethesda, MD, is kindly acknowledged for providing pS261-AQP2 antibodies.

**GRANTS**

P. M. T. Deen is a recipient of VICI grant 865.07.002 of the Netherlands Organization for Scientific research (NWO). This study was supported by grants from the Dutch Kidney Foundation (C03-2060), NWO (865.07.002), the Coordination Theme 1 (Health) of the European Community’s 7th Framework Program (HEALTH-F2-2007-201590, entitled EUFUNERON) and (RTN austaglycoporins; number 035995-2), and the UMC St. Radboud (2004-55) to P. M. T. Deen.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


