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Bidirectional regulation of AQP2 trafficking and recycling: involvement of AQP2-S256 phosphorylation

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AQP2 trafficking and recycling: involvement of AQP2-S256 phosphorylation. Am J Physiol Renal Physiol 288: F930–F938, 2005. First published December 29, 2004; doi:10.1152/ajprenal.00291.2004.—The present study examined the role of PKA and serine256 (S256) phosphorylation for AQP2 trafficking and recycling using cells transfected with wild-type AQP2 (AQP2-WT) or mutant AQP2 and high-resolution confocal microscopic techniques. In transiently transfected MDCK-C7 cells, stimulation with forskolin induced translocation of AQP2-WT to the plasma membrane. Treatment of AQP2-WT cells with the PKA inhibitor H-89 following forskolin stimulation resulted in internalization of AQP2-WT. Moreover, H-89 treatment of AQP2-S256D (mimicking constitutively phosphorylated AQP2 and hence localized to the plasma membrane) resulted in redistribution of AQP2-S256D to intracellular vesicles, even in the presence of forskolin. Both PGE2 and dopamine stimulation induced endocytosis of AQP2-WT and AQP2-S256D, respectively, in forskolin-stimulated cells. Consistent with this, dopamine in the presence of vasopressin stimulated endocytosis of AQP2 in slices of rat kidney inner medulla without substantial dephosphorylation. In conclusion, these results strongly suggest that 1) S256 phosphorylation is necessary but not sufficient for AQP2 plasma membrane expression, 2) active PKA is required for AQP2 plasma membrane expression, 3) PGE2 and dopamine induce internalization of AQP2 independently of AQP2 dephosphorylation, and 4) preceding activation of cAMP production is necessary for PGE2 and dopamine to cause AQP2 internalization.

AQUAPORINS (AQPS) ARE A FAMILY OF PLASMA MEMBRANE WATER CHANNEL PROTEINS FACILITATING MOVEMENT OF WATER ACROSS CELL MEMBRANES OF DIVERSE ORGANS. AQPS (AQP1–AQP12) ARE INVOLVED IN A WIDE RANGE OF PHYSIOLOGICAL PROCESSES. AQPS ARE EXPRESSED IN THE KIDNEY, BRAIN, SKIN, AND LIVER. AQPS ARE METABOLICALLY ACTIVE PROTEINS, AND THEIR EXPRESSION AND FUNCTION ARE MODULATED BY A VARIETY OF FACTORS, INCLUDING PHARMACOLOGICAL AGENTS, PHYSIOLOGICAL SIGNALING PATHWAYS, AND ENVIRONMENTAL STRESSORS. AQPS ARE IMPORTANT IN WATER BALANCE, ELECTROLYTE HOMEOSTASIS, AND VARIOUS OTHER BIOLOGICAL PROCESSES. AQPS ARE TARGETS FOR THE DEVELOPMENT OF PHARMACOTHERAPEUTICS AND BIOMARKERS.

AQP2, also known as aquaporin-2, is a water channel protein expressed in the collecting ducts of the kidney. AQP2 is crucial for the regulation of water reabsorption in the kidney. Phosphorylation of AQP2 at serine 256 (S256) by protein kinase A (PKA) is essential for maintaining AQP2 in the plasma membrane. PKA phosphorylation of S256 is necessary for AQP2 to be trafficked to the plasma membrane and for it to be active in facilitating water transport. Conversely, dephosphorylation of S256 by protein phosphatase 2A (PP2A) is required for AQP2 internalization and recycling. In the presence of vasopressin, PKA is activated, leading to phosphorylation of S256 and subsequent translocation of AQP2 to the plasma membrane, where it facilitates water reabsorption. Without vasopressin, AQP2 is in an internal pool, and PKA activity is low. In the presence of vasopressin escape, the inappropriately high levels of AVP are counteracted, leading to an increase in urine volume and a downregulation of AQP2 expression (17), which indicates the presence of AVP-independent regulation of AQP2.

It is hypothesized that S256 phosphorylation is necessary and sufficient for AQP2 to remain in the plasma membrane. PGE2, however, has been shown to inhibit cAMP-induced water permeability of rat terminal inner medullary collecting duct (10) and to counteract AVP-induced AQP2 translocation to the plasma membrane in slices of inner medulla (IM) independently of dephosphorylation (20), possibly via EP3 receptors (15). These findings indicate a role for PGE2 in AQP2 shuttling. Dopamine has also been shown to counteract AVP-induced water permeability in the rat cortical collecting duct, probably via D4 receptors (12) and in the inner medullary collecting duct via α2-adrenoceptors (3); still, dopamine’s action on AQP2 trafficking remains unknown.

It is well established that phosphorylation of S256 in AQP2 is necessary for translocation of AQP2 to the plasma membrane; however, whether phosphorylation of S256 is also sufficient to maintain AQP2 plasma membrane localization is unknown. This study was designed to evaluate the importance of active PKA for AQP2 plasma membrane localization and to test the hypothesis that phosphorylation of S256 is sufficient to retain AQP2 in the plasma membrane. We used a tissue culture system with Madin-Darby canine kidney (MDCK)-C7 cells transiently transfected with either AQP2 wild-type (AQP2-WT), which, on stimulation with the cAMP-elevating agent forskolin, translocated to the plasma membrane, or with the...
mutant AQP2-S256D, which mimics constitutively phosphorylated AQP2 and, hence, is localized in plasma membrane without prior forskolin stimulation (6). We used this system further to test the necessity of active PKA for AQP2 plasma membrane localization and tested the effects of PGE₂ and dopamine on AQP2 distribution. To further investigate the effect of dopamine on AQP2 localization, we also studied AQP2 distribution and phosphorylation in slices of rat IM using two types of anti-AQP2 antibodies: an antibody recognizing total AQP2 and an antibody exclusively recognizing S256 phosphorylated AQP2.

MATERIALS AND METHODS

Constructs

pCDNAI/Neo with rat cDNA encoding AQP2 (tagged with a COOH-terminal c-myc epitope) was generously provided by Dr. D. Brown (9). Point mutations were introduced using a QuickChange XL Site-Directed Mutagenesis Kit (Strategene) and the following primers: S256A sense: GCCGCGGCGCGGCGGCTTGGAGCTCC; S256A antisense: GGAGCTACCCGTCCGCGGC; S256D sense: GTGGCGGCGGGCGAGTGGAGCTC; and S256D antisense: GAGTGGAGCCTCAATCTCGGCGGCGGCAC.

Underlined letters indicate mutations. Mutations were verified by sequencing (Lark Technologies, Essex, UK).

Cell Culture

MDCK-C7 cells (18) (a gift from H. Oberleithner) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO₂. MDCK-C7 cells were grown on coverslips and transiently transfected using Effectene Transfection Reagent (Qiagen). Cells were kept in serum-free media in the continuous presence of indomethacin (10 μM) overnight before experiments and analyzed 48 h posttransfection. In preliminary experiments, the absence of indomethacin resulted in an increased AVP/forskolin-induced plasma membrane targeting of AQP2-WT, which is absent in the presence of indomethacin. Rats were treated with indomethacin (1 mg/100 g body wt, Confortid, Dumex) at 16 and 1 h before the experiment to suppress the endogenous production of prostaglandins. Rats were anesthetized with thiobutabarbital (8 mg/100 g body wt), the kidneys were rapidly removed, and the IM was excised and processed immediately as previously described (20). Briefly, IM tissue from each rat was divided into four parts, which were incubated in 1) PBS for 30 min (control); 2) PBS with AVP (10⁻⁵ M, Sigma) for 30 min; 3) PBS with AVP (10⁻⁵ M) for 30 min and dopamine (10⁻⁵ M, Intropin, DaPont Pharmaceuticals), which was added 15 min after the beginning of AVP incubation (AVP, dopamine); or 4) PBS for 15 min and dopamine (10⁻⁵ M) for 15 min (dopamine). For analysis of AQP2 phosphorylation, the tissue was homogenized in buffer A (in mM: 300 sucrose, 25 imidazole, 1 EDTA, pH 7.2, 1 phenylmethylsulfonyl fluoride, 25 sodium fluoride, and 1 sodium orthovanadate, as well as 5 μg/ml leupeptin and 0.1 μM okadaic acid). For analysis of AQP2 distribution, the IM tissue was homogenized in buffer A containing 20 μg/ml leupeptin, 20 μg/ml antipain, 5 μg/ml pepstatin A, 5 μg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride, 25 mM benzamidine, 25 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.1 μM okadaic acid (buffer B) and fractionated by differential centrifugation to generate samples enriched for either plasma membrane or intracellular vesicles. For that, the tissue homogenates were centrifuged at 4,000 g for 10 min, and the pellet containing nuclei and cell debris was discarded. The supernatant was centrifuged at 17,000 g for 30 min. The pellet was resuspended in buffer B and considered as the plasma membrane-enriched fraction. The supernatant was centrifuged at 200,000 g for 1 h. The pellet was resuspended in buffer B and considered as the intracellular vesicle-enriched fraction. Samples were then analyzed by SDS-PAGE followed by immunoblotting and densitometric analysis as previously described (20).

Data analysis and statistics. Data are presented as means ± SE. Statistical analyses were made using a paired Student’s t-test. A difference of P < 0.05 was considered statistically significant.

RESULTS

Evaluation of MDCK-C7 Cell System

The MDCK-C7 cell line was chosen to study AQP2 distribution since this clone, originally derived from the WT MDCK cells, closely resembles the principal cells of the kidney collecting duct (18). To test the distribution and trafficking of AQP2 in MDCK-C7 cells, the cells were transiently transfected with AQP2-WT, AQP2-S256A, and AQP2-S256D con-
structs, the last two mimicking constitutively nonphosphorylated and phosphorylated AQP2, respectively. Confocal laser microscopy was used to analyze the subcellular localization and trafficking, consistent with previous studies (1, 6–8, 13–16). In nontreated cells cultured in the presence of indomethacin, AQP2-WT was located in intracellular vesicles (Fig. 1A). Without indomethacin, some AQP2-WT was also located in the plasma membrane (not shown), consistent with previous evidence (1). Moreover, the ability of AQP2-WT to traffic to the plasma membrane in response to forskolin (to increase cAMP production) was also tested. AQP2-WT was translocated to the plasma membrane on stimulation with forskolin (Fig. 1B) as previously shown (1, 7, 8). In MDCK-C7 cells, AQP2-S256A was located in intracellular vesicles (Fig. 1C) and did not traffic to the plasma membrane on forskolin stimulation (not shown) as shown previously (7). Under basal conditions, AQP2-S256D was mainly localized in the plasma membrane with only little labeling of intracellular vesicles (Fig. 1D), consistent with previous results (6).

Thus based on the series of control experiments, the behavior of AQP2-WT in transiently transfected MDCK-C7 cells mimics the AVP-regulated AQP2 trafficking found in collecting duct cells and in WT MDCK cells (1), and the two mutants, AQP2-S256A and AQP2-S256D, mimic the nonphosphorylated and phosphorylated form of AQP2, respectively. Therefore, the transfected MDCK-C7 cells constitute a tissue culture model that can be used to study mechanisms of AQP2 distribution.

**PKA Inhibitor H-89 Induced Internalization of AQP2-S256D and AQP2-WT in the Presence of Forskolin**

To investigate the role of S256 phosphorylation in AQP2 endocytosis and recycling, cells transfected with AQP-WT cells were first treated with forskolin to translocate AQP2-WT to the plasma membrane (Fig. 2A) and thereafter treated with the PKA inhibitor H-89 (30 μM) for 1 h in the continuous presence of forskolin. This resulted in a marked redistribution of AQP2-WT, from a plasma membrane localization to almost exclusive intracellular localization (Fig. 2B), despite continuous forskolin stimulation. This indicated that the recycling of AQP2 is dependent on PKA activity.

To investigate whether AQP2 could be endocytosed independently of S256 dephosphorylation, the AQP2-S256D mutant mimicking constitutively phosphorylated AQP2 was studied. Under basal conditions, the AQP2-S256D mutant was almost exclusively localized to the plasma membrane (Fig. 2C). H-89 treatment of AQP2-S256D-transfected cells (Fig. 2, D and E) resulted in internalization of AQP2-S256D in both the continuous presence of forskolin (Fig. 2D) and the absence of forskolin (Fig. 2E), demonstrating that dephosphorylation of S256 is not necessary for AQP2 endocytosis. Moreover, this
indicates that S256 phosphorylation is not sufficient to maintain AQP2 in the plasma membrane and indicates that other proteins involved in AQP2 endo- and exocytosis are regulated by PKA.

**PGE2 Induced Intracellular Distribution of AQP2-S256D and AQP2-WT in the Continuous Presence of Forskolin**

PGE2 counteracts AVP-induced increase in collecting duct water permeability (10), probably by inducing a redistribution of AQP2 from the plasma membrane to intracellular vesicles (20). To further elucidate the role of PGE2 in AQP2 trafficking, MDCK-C7 cells transiently transfected with AQP2-WT and AQP2-S256D were treated with PGE2, either with or without prior forskolin stimulation.

As shown above, under basal conditions, AQP2-WT was located intracellularly (Fig. 1A). After forskolin stimulation, AQP2-WT was inserted into the plasma membrane (Fig. 3A). This insertion was reversed by PGE2 stimulation (Fig. 3B), indicating that PGE2 can reverse cAMP-mediated AQP2 plasma membrane distribution. To investigate whether PGE2-mediated endocytosis of AQP2 can occur independently of S256 dephosphorylation, the AQP2-S256D mutant mimicking constitutively phosphorylated AQP2 was studied. Under basal conditions, AQP2-S256D was located to the plasma membrane (Fig. 3C). PGE2 stimulation did not alter the distribution of AQP2-S256D (Fig. 3D), whereas PGE2 stimulation after forskolin stimulation resulted in internalization of AQP2-S256D (Fig. 3E), demonstrating that dephosphorylation of S256 is not
necessary for PGE2-mediated AQP2 endocytosis, but activation of cAMP production is.

These results further support the findings that S256 phosphorylation is not sufficient for AQP2 plasma membrane distribution and that AQP2 endocytosis can occur completely independent of S256 dephosphorylation.

Dopamine Induces Endocytosis of AQP2-WT and AQP2-S256D When cAMP Production Is Activated

Dopamine, like PGE$_2$, is also known to reverse AVP-induced water permeability of the kidney collecting duct (12). However, the mechanism of this counteraction is unknown. To investigate whether the counteracting mechanism of dopamine is associated with a redistribution of AQP2 protein from the plasma membrane to intracellular vesicles, we investigated the effect of dopamine on the subcellular distribution of AQP2-WT and AQP2-S256D in MDCK-C7 cells.

We found that forskolin-stimulated plasma membrane insertion of AQP2-WT (Fig. 4A) was reversed by dopamine stimulation in the continuous presence of forskolin, resulting in intracellular distribution of AQP2-S256D, which was still localized in the plasma membrane after PGE$_2$ stimulation (arrows).
AQP2 distribution in the cells transiently transfected with the mutant AQP2-S256D mimicking constitutively phosphorylated AQP2, hence, as described above, localized to the plasma membrane without activation of cAMP production. Dopamine alone (Fig. 4E) did not alter AQP2-S256D plasma membrane localization (Fig. 4C), whereas dopamine stimulation following forskolin stimulation resulted in internalization of AQP2-S256D (Fig. 4D), demonstrating that dephosphorylation of S256 is not necessary for dopamine-mediated AQP2 endocytosis, but activation of cAMP production is.

Dopamine Reversed AVP-Induced Redistribution of AQP2 in Slices From Rat IM Tissue

The role of dopamine on AQP2 trafficking was further examined using rat IM slices (Fig. 5, A and B). Stimulation of slices with AVP, as expected, shifted AQP2 from the fraction enriched for intracellular vesicles to the fraction enriched for plasma membrane and hence altered the plasma membrane-to-intracellular vesicle ratio of AQP2 (Fig. 5B, controls 100% vs. AVP 139 ± 11%). When slices were stimulated with dopamine following AVP stimulation, the membrane-to-intracellular vesicle ratio of AQP2 was significantly reduced (Fig. 5B, AVP 139 ± 11% vs. AVP+dopamine 108 ± 11%) and did not differ from the control level. Dopamine alone did not significantly change the membrane-to-intracellular vesicle ratio of AQP2 abundance (Fig. 5B, controls 100% vs. dopamine 91 ± 6%). These results suggest that dopamine counteracts AVP-induced water permeability in the renal collecting duct by a redistribution of AQP2 from the plasma membrane to intracellular vesicles. Using a phospho-specific antibody exclusively...
DISCUSSION

In this study we investigated the importance of S256 phosphorylation/dephosphorylation for AQP2 plasma membrane localization and recycling using cells transfected with either AQP2-WT or the mutant AQP2-S256D, which mimics constitutively phosphorylated AQP2 and, hence, localized to the plasma membrane even in the absence of cAMP stimulation. The results demonstrated that inhibition of PKA by H-89 resulted in redistribution of the mutant AQP2-S256D from the plasma membrane to intracellular vesicles, indicating that active PKA is necessary for maintaining AQP2 in the plasma membrane even when S256 is mutated to S256D, which mimics constitutively phosphorylated AQP2. We also studied the effect of PGE2 and dopamine, and both markedly reversed forskolin-stimulated plasma membrane expression of AQP2-WT, leading to an exclusive intracellular expression. Moreover, PGE2 or dopamine stimulation resulted in internalization of the mutant AQP2-S256D from the plasma membrane to intracellular vesicles. Importantly, this effect was only observed when cAMP production was activated by forskolin. In addition, we tested the effect of dopamine on AQP2 distribution and phosphorylation using slices of rat IM tissue. We found that dopamine reverses AVP-induced redistribution of AQP2 and that a substantial fraction of AQP2 remained phosphorylated on S256 on internalization. Thus this strongly indicates that the physiologically well established reduction in the osmotic water permeability seen by PGE2 or dopamine in reversing the vasopressin-induced water permeability is caused by internalization of AQP2 from the plasma membrane to intracellular vesicles and that this effect is dependent on activation of cAMP and PKA.

Methodological Considerations

Control experiments using WT and mutant AQP2 in various experimental conditions (treatment with vasopressin or forskolin) revealed results identical to those in previous studies (1, 6, 7, 8, 13–16) using confocal laser microscopy to analyze the subcellular localization and trafficking. Although confocal microscopy does not allow a resolution to document, e.g., plasma membrane localization, the method has proven valid in previous investigations within the same or similar areas. It should also be emphasized that studies based on tissue culture systems may only in part reflect in vivo conditions. Importantly, the effects seen on AQP2 trafficking parallels nicely expected observations based on previous physiological in vivo or renal tubule perfusion studies. This is discussed below.

Active PKA is Necessary for AQP2 Membrane Localization

Our results indicate that AQP2 distribution to the plasma membrane is dependent not only on phosphorylation on S256 but also on active PKA, strongly indicating that PKA-mediated phosphorylation of other proteins is essential in vasopressin-induced AQP2 plasma membrane targeting. Conversely, this also demonstrates that S256 phosphorylation is not sufficient to maintain AQP2 in the plasma membrane. In further support of this, the results also revealed that AQP2 redistribution from the plasma membrane to intracellular vesicles can occur independently of dephosphorylation of S256. Thus cAMP/PKA is involved in bidirectional trafficking of AQP2 by being in-

recognizing phosphorylated AQP2 we showed that dopamine stimulation was associated with a decrease in AQP2 phosphorylation (Fig 5, C and D). Both basal levels and AVP-induced phosphorylation of AQP2 were decreased by dopamine (Fig. 5D, controls 100% vs. AVP 169 ± 4%, AVP 169 ± 4% vs. AVP+dopamine 133 ± 5%, controls 100% vs. dopamine 67 ± 5%); however, dopamine stimulation did not completely abolish the AVP-induced increase in AQP2 phosphorylation. These findings, like the results found with H-89 and PGE2, support the notion that S256 phosphorylation may be necessary but not sufficient for AQP2 plasma membrane localization and that AQP2 can be endocytosed independently of S256 dephosphorylation.

Fig. 5. Dopamine induces AVP-dependent endocytosis of AQP2 in inner medullary slices. Immunoblot from inner medullary slices treated with AVP, dopamine, or in combination and densitometric analysis. A: immunoblot of total AQP2 in plasma membrane-enriched fractions (M) and fractions enriched for intracellular vesicles (V). B: there was a significant increase in the AQP2 M/V ratio in AVP-treated slices compared with controls. Dopamine stimulation following AVP stimulation induced a significant reduction in this ratio. Dopamine alone did not alter the AQP2 M/V ratio compared with controls. C: immunoblot of AQP2 phosphorylation at S256 visualized using phosphorylated state-specific AQP2 antibody (p-AQP2). Con, control; Dop, dopamine. D: there was a significant increase in p-AQP2 levels in AVP-treated inner medullary slices compared with controls. Dopamine stimulation following AVP treatment induced a significant reduction in p-AQP2; however, p-AQP2 was still significantly increased compared with controls. Dopamine alone decreased p-AQP2 levels compared with controls.
Importance of S256 Phosphorylation for AQP2 Plasma Membrane Expression

PKA phosphorylation of three or four of the four monomers of AQP2 at S256 is associated with AQP2-bearing vesicles being exocytosed and AQP2 being inserted into the plasma membrane (6). It has generally been hypothesized and accepted that AQP2 endocytosis is mediated by dephosphorylation of S256 based on the observation that the AQP2-S256A mutant (mimicking constitutively dephosphorylated AQP2) is localized intracellularly and does not traffic to the plasma membrane on forskolin/cAMP stimulation (7) and on observations showing that vasopressin V2-receptor antagonist treatment of rats both reduces AQP2 phosphorylation, increases internalization of AQP2, and produces severe polyuria. Moreover, it was generally believed that PKA phosphorylation of S256 is not only necessary but also sufficient for AQP2 to remain in the plasma membrane. However, it was recently shown that PGE2 treatment of AVP-stimulated slices of rat IM tissue resulted in redistribution of AQP2 from the plasma membrane-enriched fraction to the fraction enriched with intracellular vesicles, which was not associated with significant dephosphorylation of AQP2 (20). It has also been shown that the PKC activator TPA, which is known to induce a general endocytosis in MDCK cells (5), induced endocytosis of AQP2-S256D, indicating that AQP2 can be endocytosed without prior dephosphorylation (16). However, it remains unknown whether this endocytosis is AQP2 specific or merely a part of the PKC-activated general endocytosis. The present work aimed to obtain more direct evidence regarding the necessity and sufficiency of S256 phosphorylation and dephosphorylation for AQP2 plasma membrane distribution and also to test the role of active PKA for AQP2 plasma membrane distribution. For this purpose, we used MDCK-C7 cells transiently transfected with either AQP2-WT or the mutant AQP2-S256D, mimicking constitutively phosphorylated AQP2, which is localized to the plasma membrane even without activation of cAMP production and hence provides a unique model to study AQP2 dephosphorylation-independent distribution.

Dephosphorylation of AQP2 at S256 is Not Necessary for AQP2 Internalization

We found that inhibition of PKA by the inhibitor H-89 induced redistribution of AQP2-S256D and forskolin-stimulated AQP2-WT from plasma membrane localization to intracellular compartments. This redistribution occurred despite the fact that the mutant AQP2-S256D is not a target for dephosphorylation (and hence cannot be dephosphorylated) and constitutively carries a negative charge at the S256 position. This indicates insufficiency of S256 phosphorylation to maintain AQP2 in the plasma membrane. This also indicates that for AQP2 plasma membrane localization, PKA is not only necessary for direct phosphorylation of S256 in AQP2 but is also essential in regulating other proteins that control AQP2 shuttling. Several lines of evidence indicate that activation of PKA deactivates RhoA, causing actin depolymerization and AQP2 exocytosis (14). If RhoA is not deactivated by PKA, PKA-mediated AQP2 exocytosis is impaired (13). This deactivation could also be involved in the regulation of AQP2 localization in the steady state. These results provide novel evidence that S256 phosphorylation of AQP2 is necessary but not sufficient for AQP2 localization to the plasma membrane and that other proteins must be activated or deactivated by PKA for AQP2 plasma membrane localization.

Mechanisms of Vasopressin Escape

The results of the present study may have significant impact on the understanding of a series of physiological and pathological conditions of vasopressin escape, syndrome of inappropriate antidiuretic hormone (vasopressin) secretion, conditions with water and sodium retention (liver cirrhosis, congestive heart failure), as well as conditions where dopamine and PGE2 modulate renal water reabsorption. This relates to the results regarding the bidirectional regulation of AQP2 trafficking being dependent on AQP2 phosphorylation as well as the results delineating the cellular mechanisms behind the effects of PGE2 and dopamine (namely, cAMP-dependent AQP2 and AQP2-S256D internalization). Vasopressin escape is characterized by a sudden increase in urine volume with a decrease in urine osmolality despite high circulating levels of AVP. The onset of vasopressin escape coincides with a marked decrease in the expression level and plasma membrane targeting of AQP2 (17); however, the cellular mechanisms of vasopressin escape are unknown. Both PGE2 and dopamine are known to counteract AVP-induced water permeability of the kidney collecting duct (6, 9) and therefore could potentially be involved in the vasopressin escape mechanisms.

We found that PGE2 and dopamine mediated an alteration in the localization of forskolin-stimulated AQP2-WT from the plasma membrane to intracellular vesicles. We also found that dopamine induced a shift in the presence of AQP2 from plasma membrane-enriched fractions to intracellular vesicle-enriched fractions of AVP-stimulated IM slices. These results are consistent with the ability of PGE2 and dopamine to counteract AVP-induced collecting duct water permeability.

Interestingly, PGE2 and dopamine did not significantly alter the plasma membrane localization of AQP2-S256D without prior forskolin stimulation. This is consistent with previous physiological data (10). It should be mentioned that it has been reported that PGE2 may have direct effects in stimulating water permeability and that there is increased plasma membrane AQP2 targeting in the absence of indomethacin treatment of MDCK cells transfected with AQP2 (Ref. 1 and present data). However, most studies in mammalian kidney cells or renal tubules show an absence of changes in water permeability...
directly by PGE$_2$ alone but only after vasopressin, forskolin, or even cAMP analogs (10). The present results are consistent with this notion since no change in AQP2 subcellular localization was observed with treatment of PGE$_2$ or dopamine alone (in the absence of forskolin). When cells were pretreated with forskolin, AQP2-S256D was internalized from the plasma membrane to intracellular vesicles on PGE$_2$ or dopamine stimulation. In IM slices, dopamine stimulation was associated with a marked decrease in AQP2 phosphorylation. However, a large fraction of AQP2 remained phosphorylated on the internalization. Our tissue culture data suggest that the dephosphorylation may take place after redistribution of AQP2 from the plasma membrane to intracellular vesicles. In the same IM tissue slice setup, PGE$_2$ stimulation alone or following AVP stimulation was not associated with a decrease in AQP2 phosphorylation (20), supporting this hypothesis. The ability of PGE$_2$ and dopamine to counteract the plasma membrane localization of AQP2-S256D, mimicking constitutively phosphorylated AQP2, supports the hypothesis that PGE$_2$ and dopamine may be involved in the vasopressin escape mechanism. Whether AQP2 dephosphorylation occurs during or after endocytosis in vivo is still unknown. However, the ability to induce AQP2 endocytosis without prior dephosphorylation would allow a very rapid response to diuretic stimuli and would be especially useful in conditions like the vasopressin escape, where a high level of phosphorylated AQP2 is expected due to constitutive high AVP levels.

In summary, our results suggest that 1) phosphorylation of S256 is necessary but not sufficient for AQP2 plasma membrane localization; 2) active PKA is required for AQP2 plasma membrane localization; 3) PGE$_2$ and dopamine are able to induce a redistribution of AQP2 from the plasma membrane to intracellular vesicles without prior AQP2 dephosphorylation; and 4) preceding activation of cAMP production is necessary for PGE$_2$ and dopamine to cause AQP2 internalization.

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