Localization and regulation of PKA-phosphorylated AQP2 in response to V2-receptor agonist/antagonist treatment

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Am. J. Physiol. Renal Physiol. 278: F29–F42, 2000.—Phosphorylation of Ser256, in a PKA consensus site, in AQP2 (p-AQP2) appears to be critically involved in the vasopressin-induced trafficking of AQP2. In the present study, affinity-purified antibodies that selectively recognize AQP2 phosphorylated at Ser256 were developed. These antibodies were used to determine 1) the subcellular localization of p-AQP2 in rat kidney and 2) changes in distribution and/or levels of p-AQP2 in response to [desamino-Cys5, d-Arg8]vasopressin (DDAVP) treatment or V2-receptor blockade. Immunoelectron microscopy revealed that p-AQP2 was localized in both the apical plasma membrane and in intracellular vesicles of collecting duct principal cells. Treatment of rats with V2-receptor antagonist for 30 min resulted in almost complete disappearance of p-AQP2 labeling of the apical plasma membrane with only marginal labeling of intracellular vesicles remaining. Immunoblotting confirmed a marked decrease in p-AQP2 levels. In control Brattleboro rats (BB), lacking vasopressin secretion, p-AQP2 labeling was almost exclusively present in intracellular vesicles. Treatment of BB rats with DDAVP for 2 h induced a 10-fold increase in p-AQP2 labeling of the apical plasma membrane. The overall abundance of p-AQP2, however, was not increased, as determined both by immunoelectron microscopy and immunoblotting. Consistent with this, 2 h of DDAVP treatment of normal rats also resulted in unchanged p-AQP2 levels. Thus the results demonstrate that AQP2 phosphorylated in Ser256 is present in the apical plasma membrane and in intracellular vesicles and that both the intracellular distribution/trafficking, as well as the abundance of p-AQP2, are regulated via V2 receptors by altering phosphorylation and/or dephosphorylation of Ser256 in AQP2.

aquaporin-2; phosphorylation; vasopressin

Vasopressin regulates collecting duct water reabsorption predominantly, if not exclusively, through regulated trafficking of aquaporin-2 (AQP2) from intracellular vesicles to the apical plasma membrane (35, 42, 48, 54). This acute response occurs within a few minutes and is mediated by binding of vasopressin to V2 receptors situated in the basolateral membrane of collecting duct principal cells. The synthesis of cAMP is subsequently stimulated via a V2-receptor-coupled G protein, which activates adenylate cyclase. Protein kinase A (PKA) is a multimeric protein which is activated by cAMP, and early studies have demonstrated that PKA induces phosphorylation of various membrane proteins in bovine kidney (10), and in saponin-permeabilized outer medullary collecting duct segments phosphorylation of at least two unidentified 45- and 66-kDa proteins have been demonstrated (17). Following PKA phosphorylation, intracellular vesicles containing AQP2 are transferred to the membrane possible by the involvement of dynein and dynactin, which have been shown to colocalize with AQP2-bearing vesicles in collecting duct principal cells (36). Ultimately, the vesicles fuse with the apical plasma membrane by exocytosis, which is speculated to be mediated by interaction of vesicle-targeting receptor proteins present in the vesicles (v-SNAREs) with vesicle-targeting proteins in the target membrane (t-SNAREs), e.g., VAMP-2, syntaxin-4, and SNAP-23 (20, 21, 32, 44).

The exact role of phosphorylation of AQP2 in the trafficking of AQP2 is presently not known. AQP2 contains a consensus site (Arg-Arg-Gln-Ser) for PKA phosphorylation in the cytoplasmic COOH terminus at serine-256. Recent studies have shown a very rapid phosphorylation of this serine (within 1 min) in response to vasopressin treatment of slices of kidney papilla (46). This is in agreement with the time course of vasopressin-stimulated water permeability of kidney collecting ducts (27, 53).

The water permeability of the plant water channels PM28A and α-TIP appears to be regulated by in situ phosphorylation presumably by altering the conductance (22, 38). However, PKA phosphorylation of AQP2 does not seem to have any significant effect on the osmotic water permeability. In Xenopus oocytes, cAMP-induced PKA phosphorylation of AQP2 only caused a small increase in water permeability (28), and in AQP2-containing vesicles purified from kidney inner medulla no increase in water permeability was seen upon PKA stimulation (30). PKA-induced phosphorylation of AQP2 may instead be required to modulate the distribution of AQP2 between plasma membrane and intracellular vesicle compartments as recently indicated. Both cAMP (14) and vasopressin or forskolin
treatment (25) failed to induce translocation of AQP2 in LLC-PK₁ cells when Ser²⁵⁶ was substituted by an alanine in contrast to normal trafficking of wild-type AQP2, highlighting the importance of the PKA consensus site for regulated and/or constitutive trafficking.

To investigate the role of phosphorylation of AQP2 in Ser²⁵⁶, we have used antibodies that selectively recognize AQP2 phosphorylated in this PKA consensus site and antibodies that recognize both phosphorylated and nonphosphorylated AQP2. The antibodies were used to determine 1) the subcellular localization of phosphorylated AQP2 in normal rats and in vasopressin-deficient Brattleboro rats, 2) the effect of V₂-receptor antagonist treatment on phosphorylated AQP2 protein abundance and distribution in normal rats, and 3) the effect of DDAVP treatment on the abundance and distribution of phosphorylated AQP2 in Brattleboro rat kidneys.

MATERIALS AND METHODS

Experimental Animals

Wistar rats were obtained from Møllegaard Breeding Center (Ejby, Denmark), and homozygous Brattleboro rats were from Harlan Nederland (Horst, The Netherlands). During experiments rats, were placed in metabolic cages with free access to standard rat diet and water. The following protocols were used.

Experimental Protocols

Protocol 1. Normal rats were treated intravenously with V₂-receptor antagonist 5-dimethylamino-1-(4-(2-methylbenzoylamino)benzoyl)-2,3,4,5-tetrahydro-1H-benzazepine (OPC-31260; Otsuka, Tokyo, Japan) through the femoral vein (1 mg in 0.2 ml vehicle/animal, n = 4 rats) (7). Control rats received intravenous saline (n = 4 rats). During the procedure, the animals were briefly anesthetized with isofluran. After 30 min of the injection, anesthesia was repeated and kidneys were either removed for preparation of membrane fractions or perfusion fixed for immunocytochemistry.

Protocol 2. Normal rats were treated intravenously with OPC-31260 (1 mg in 0.2 ml vehicle/animal, n = 4 rats) (7). Control rats received intravenous saline (n = 4 rats). During the procedure, the animals were briefly anesthetized with isofluran. After 60 min of the injection, anesthesia was repeated and kidneys were removed for preparation of membrane fractions.

Protocol 3. Brattleboro rats were treated subcutaneously with DDAVP ([desamino-Cys⁵⁰,p-Arg⁶⁰]vasopressin, 50–1,000 ng in 500 μl saline/animal, n = 8 rats; Sigma-Aldrich, Vallensbaek Strand, Denmark). Control rats received subcutaneous saline (n = 7 rats). After 2 h of the injection, anesthesia was repeated and kidneys were either removed for preparation of membrane fractions (4 control rats and 4 DDAVP-treated rats) or perfusion fixed for immunocytochemistry (3 control rats and 4 DDAVP-treated rats).

Protocol 4. Normal rats were treated subcutaneously with DDAVP (500 ng in 500 μl saline/animal, n = 4 rats). Control rats received subcutaneous saline (n = 3 rats). After 2 h of the injection, anesthesia was repeated and kidneys were removed for preparation of membrane fractions.

Antibodies

For preparation of anti-phosphorylated AQP2 antibodies (anti-p-AQP2), two peptides corresponding to amino acids 253–262 of rat AQP2 were synthesized with addition of a cysteine residue at the carboxyl terminus and a glycine residue at the amino terminus (peptides 3 and 4, Table 1). Both peptides were phosphorylated at Ser²⁵⁶ in the PKA phosphorylation site (Arg-Arg-Gln-Ser). In one of the peptides, amino acids 259 (leucine) and 260 (histidine) were swapped to reduce production of clones of antibodies recognizing nonphosphorylated aquaporin-2 (AQP2).

Affinity purification of anti-AQP2 antibody (LL127) (raised against a peptide corresponding to amino acids 250–271 of rat AQP2, peptide 1; Table 1) was performed as previously described (43). Immunoblotting (Fig. 1) demonstrated that this AQP2 antibody recognizes both nonphosphorylated and phosphorylated AQP2 peptides (amino acids 250–271). There are no significant changes in the affinity for the two peptides.

Immunocytochemistry

Immunocytochemistry was performed essentially as previously described (7, 41, 42). Kidneys were perfusion fixed with either 1) 2% paraformaldehyde in PLP (0.01 M NaOH, 0.075 M L-lysine, and 0.0375 M Na₂HPO₄, pH 6.2); or 2) 0.1% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4; or 3) 0.2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4, via the abdominal aorta. Tissue blocks were prepared from kidney inner medulla or inner stripe outer medulla. The blocks were infiltrated with 2.3 M sucrose/2% paraformaldehyde for 30 min, mounted on holders, and rapidly frozen in liquid nitrogen. Frozen tissue blocks were either directly used for cryosectioning or subjected to a cryosubstitution and Lowicryl HM20 embedding prior to ultramicrotomy. Cryosubstitution was performed as described previously (37, 45). The frozen samples were freeze-substituted in a Reichert AF5 (Reichert, Vienna, Austria). Samples were sequentially equilibrated over 3 days.
Electron micrographs were printed at a final magnification of \( \times 63,000 \). The total area of the cytoplasm was determined using a lattice square test system with a size of the squares of 30 mm \( \times \) 30 mm. The total labeling density was calculated as the total number of gold particles per cytoplasm area. To assure that the immunogold quantitation was performed on comparable tissue sampling, the length of the apical plasma membrane and the area of the cell (excluding nuclei) were determined. The lengths of the apical plasma membrane as a fraction of the area of cytoplasm in untreated and DDAVP-treated Brattleboro rats were 0.21 \( \pm \) 0.04 and 0.23 \( \pm \) 0.01, respectively. In untreated and in OPC-31260-treated rats, the values were 0.17 \( \pm \) 0.01 and 0.16 \( \pm \) 0.01, respectively. This indicates equal sampling, i.e., same ratio of apical surface to cytoplasmic area for both set of animals. The length of the apical plasma membrane was determined by a manual tracing device, and the number of gold particles per length apical plasma membrane was calculated. The number of gold particles associated with apical plasma membranes, intracellular vesicles, and multivesicular bodies was determined. Gold particles in structures that could not be identified distinctly as vesicles or multivesicular bodies or apical plasma membrane were counted separately. This is likely to represent labeling of tangentially sectioned vesicles or parts of the rough endoplasmic reticulum. The total labeling density was also determined in the apical part of inner medullary collecting duct principal cells in normal rats treated with OPC-31260 for 30 min (iv) (\( n = 4 \)) and corresponding control animals receiving saline treatment (iv) for 30 min (\( n = 4 \)).

Membrane Fractionation for Immunoblotting

The inner medulla was dissected from each kidney, minced finely, and homogenized in 2 ml of dissecting buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2, and containing the protease and phosphatase inhibitors 8.5 \( \mu \)M leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 mM oka acid, 1 mM sodium orthovanadate, and 25 mM sodium fluoride) (34, 35, 46). This homogenate was centrifuged in a Beckman L8M centrifuge at 4,000 g for 15 min at 4°C. The supernatant was centrifuged at 200,000 g for 1 h, and the resultant pellet, containing a mixture of plasma membranes and intracellular vesicles, was resuspended in dissecting buffer.

Electrophoresis and Immunoblotting

Samples of membrane fractions from inner medulla were subjected to SDS-PAGE (29) using 12% polyacrylamide mini-gels (Bio-Rad Mini Protein II) and transferred to nitrocellulose paper by electroelution. Blots were blocked with 5% milk in PBS-T (80 mM Na\(_2\)HPO\(_4\), 20 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with anti-p-AQP2 or anti-AQP2 antibody. After washing in PBS-T, the blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (P448; Dako). After final washing in PBS-T, p-AQP2 or total AQP2 was visualized using the ECL enhanced chemiluminescence system (Amersham International, Buckinghamshire, UK). Immunolabeling controls using preabsorbed anti-p-AQP2 antibody revealed no labeling (data not shown).

Statistical Analysis

For densitometry of immunoblots, samples from OPC-31260- and DDAVP-treated animals were run on gels with corresponding samples from control animals. Films were scanned using a Agfa Archus II scanner and Adobe Photoshop Software. The scanning was performed using ECL.
exposures that gave bands in lower gray scale where there is a linear correlation between signal and protein levels (34). The labeling density was quantitated using specially written software (35). p-AQP2 labeling in the samples from the experimental animals was calculated as a fraction of the mean control value for that film. Both the 29-kDa and the 35- to 50-kDa band (corresponding to nonglycosylated and the glycosylated AQP2) were scanned (34, 42, 43). Values were corrected by densitometry of Coomassie-stained preliminary gels. Values are presented in the text as means ± SE. Comparisons between groups were made by unpaired t-test. P < 0.05 was considered significant.

RESULTS

Selectivity of Anti-p-AQP2 Antibody

As shown in Fig. 1, the nonselective anti-AQP2 antibody (LL127) recognized both the phosphorylated and the nonphosphorylated peptides with similar efficiency. In contrast, the affinity-purified anti-phosphorylated AQP2 antibodies selectively recognized p-AQP2 as determined by immunoblotting. Figure 2 shows the selectivity of the antibody produced from peptide 3 (Table 1). In Fig. 2A immune serum was used, and in Fig. 2B a double affinity-purified preparation was used. Both antibody preparations recognized 29- and 35- to 50-kDa bands corresponding to nonglycosylated and glycosylated AQP2 (43) and selectively recognized the phosphorylated peptide corresponding to amino acids 253–262 of rat AQP2 but not the nonphosphorylated peptide corresponding to amino acids 250–271 of rat AQP2. The same results were obtained with the antibody produced from peptide 4 (Table 1) (46). Absorption controls using preabsorbed anti-p-AQP2 antibody showed no labeling (data not shown).

Cellular and Subcellular Distribution of p-AQP2

Our previously described AQP2 antibody (9, 43) (LL127, recognizing both phosphorylated and nonphosphorylated AQP2, Fig. 1) labeled the apical plasma membrane and intracellular domains in collecting duct principal cells (Fig. 3A) consistent with previous investigations (43).

Figure 3B shows a cryosection from inner stripe of the outer medulla from a normal rat labeled with the anti-p-AQP2 antibody. Phosphorylated AQP2 was present in apical plasma membrane domains in collecting duct principal cells significantly. Labeling of intracellular domains was more pronounced using a higher concentration of the antibody (inset, Fig. 3B). A similar cellular location of p-AQP2 was found in cryosections from inner medulla from a normal rat (Fig. 3, C and D). In kidney inner medulla, p-AQP2 was located in apical plasma membrane domains (Fig. 3C), and labeling with a higher concentration of the antibody resulted in abundant labeling of intracellular domains (Fig. 3D). Immunoelectron microscopy showed prominent labeling of the apical plasma membrane (Fig. 4). In addition, there was a significant labeling associated with intracellular vesicles. Thus, in normal rats p-AQP2 is located both in the apical plasma membrane and in intracellular vesicles in collecting duct principal cells.

Effects of V2-Receptor Antagonist Treatment on p-AQP2 Levels and Intracellular Distribution in Normal Rats

To assess the effect of V2-receptor antagonist treatment on p-AQP2 protein levels in normal rats, rats were treated intravenously with OPC-31260 (1 mg) for 30 or 60 min using protocols previously described (7). OPC-31260 treatment caused a marked increase in urine production (7). Immunoblots of membrane fractions from kidney inner medulla of saline-injected control rats and OPC-31260-treated animals (Fig. 5) showed a marked reduction in p-AQP2 protein levels after 30 and 60 min of V2-receptor antagonist treatment (n = 4, P < 0.005). The reduced expression of p-AQP2 was confirmed by immunocytochemistry using thin cryosections from kidney inner medulla labeled with anti-p-AQP2 antibody (Fig. 6). A marked reduction in the overall p-AQP2 labeling was seen in rats.
Effects of DDAVP Treatment on p-AQP2 Levels and Intracellular Distribution in Brattleboro Rats

Prior to investigation of the effect of DDAVP treatment on p-AQP2 levels and intracellular distribution in Brattleboro rats, the urine production was determined. Brattleboro rats were kept in metabolic cages for 2 h after exposure to DDAVP (protocol 3). DDAVP treatment had a marked antidiuretic effect resulting in a 93% reduction in urine output (19.3 ± 1.1 vs. 1.4 ± 0.5 ml/2 h, n = 20). To investigate the subcellular distribution of p-AQP2 in Brattleboro rats and to see whether DDAVP treatment changes the subcellular distribution, immunocytochemistry was performed. In un-

1 Because of the low count of gold particles in V2-receptor antagonist-treated animals, it was not possible to quantify meaningfully and compare the changes in labeling of the apical plasma membrane and in intracellular vesicles.
treated Brattleboro rats, a distinct labeling of p-AQP2 was seen (Fig. 8A). Thus in vasopressin-deficient Brattleboro rats, significant levels of p-AQP2 are present. In untreated Brattleboro rats, p-AQP2 was almost exclusively found in intracellular domains and not in plasma membrane domains (Fig. 8A). In contrast, DDAVP treatment caused a marked increase in the labeling of apical plasma membrane domains (Fig. 8B). To further examine the subcellular distribution of p-AQP2, immunoelectron microscopy was carried out. A distinct p-AQP2 labeling of intracellular vesicles was observed in untreated Brattleboro rats, whereas low labeling was associated with the apical plasma membrane (Fig. 9A). In contrast, there was an extensive labeling of the apical plasma membrane in response to DDAVP treatment (Fig. 9B). The changes in the subcellular distribution of p-AQP2 in response to DDAVP treatment were additionally confirmed by semi-quantitation of the immunogold labeling in the apical part of collecting duct principal cells (Table 2). The fraction of total p-AQP2 labeling in the apical plasma membrane was only $0.022 \pm 0.007$ ($n = 3$) in control rats. After DDAVP exposure, the fraction of p-AQP2 in the apical plasma membrane was increased to $0.21 \pm 0.02$ ($n = 4$).

The immunocytochemistry (Fig. 8) indicated no major changes in the levels of overall labeling. To confirm this, semi-quantitative immunoblotting and semi-quantitative immunogold labeling was undertaken. Immunoblotting showed no significant changes in p-AQP2 levels after DDAVP treatment of Brattleboro rats ($0.91 \pm 0.21$ vs. $1.00 \pm 0.18$ in controls, $n = 4$) (Fig. 10). Consistent with this, semi-quantitation of the immuno-
gold labeling of p-AQP2 revealed no significant changes in total labeling density between untreated and DDAVP-treated Brattleboro rats (Table 2). In normal rats, 2 h of DDAVP treatment also failed to increase p-AQP2 levels (0.79 ± 0.18 vs. 1.00 ± 0.15 in controls, n = 4). Thus 2 h of DDAVP treatment causes a marked increase in apical plasma membrane labeling of phosphorylated AQP2 in Brattleboro rats, but the overall abundance of p-AQP2 is not changed.

**DISCUSSION**

Considerable evidence has been provided to support the view that phosphorylation of AQP2 in Ser256 (a PKA consensus site) is critically involved in regulation of AQP2 trafficking (14, 25). However, the exact role of phosphorylation of AQP2 with respect to the onset and offset response to vasopressin remains to be identified. To further investigate the role of PKA phosphorylation of AQP2 in regulated trafficking, we have produced and characterized antibodies that either selectively recognize phosphorylated AQP2 or recognize both nonphosphorylated and phosphorylated AQP2 (with respect to the PKA consensus site). Immunocytochemistry at the light microscopic and electron microscopic level showed that p-AQP2 was present in both the apical plasma membrane and in subapical vesicles in collecting duct principal cells in normal rats. V2-receptor antagonist treatment of normal rats markedly reduced p-AQP2 levels as determined by semi-quantitative immunoblotting, and immunocytochemistry confirmed marked reduction both in intracellular vesicles and the apical plasma membrane. In vasopressin-deficient Brattleboro rats, p-AQP2 was almost exclusively present in intracellular vesicles. Two hours of DDAVP treatment induced a dramatic increase in the apical plasma membrane labeling of p-AQP2, whereas total p-AQP2 levels were not increased. Taken together, the results support the view that AQP2 is phosphorylated in intracellular vesicles and that p-AQP2 is subjected to trafficking to the apical plasma membrane in response to DDAVP treatment. Furthermore, the results demonstrate that phosphorylation and/or dephosphorylation of AQP2 is regulated via V2 receptors. The results are consistent with the hypothesis that phosphorylation of AQP2 at Ser256 is involved in regulated trafficking of AQP2 to control collecting duct water permeability but that other mechanisms may be also required to regulate the trafficking/docking/fusion of AQP2 bearing vesicles with the apical plasma membrane.

**p-AQP2 Trafficking**

In vasopressin-deficient Brattleboro rats, p-AQP2 was almost exclusively present in intracellular vesicles. Two hours of DDAVP treatment induced a 10-fold increase in the apical plasma membrane content of p-AQP2. This dramatic change in p-AQP2 labeling is consistent with previous studies on Brattleboro rats, where AQP2 has been shown to be translocated to the apical plasma membrane in response to acute or chronic vasopressin treatment (9, 48, 54). The phosphorylation of AQP2 at Ser256 and the trafficking of AQP2 can in principle take place in three ways: 1) phosphorylation of AQP2 in an intracellular compartment with subsequent vasopressin-mediated trafficking induced via other/additional signaling mechanisms; 2) vasopressin-induced phosphorylation of AQP2 in vesicles directly causing exocytosis of p-AQP2; 3) vasopressin-induced trafficking of unphosphorylated AQP2 followed by phosphorylation in the apical plasma membrane and subsequent internalization of p-AQP2. These possibilities will be discussed in the following.

One mechanism could potentially involve trafficking of AQP2 residing in a phosphorylated state in intracellular vesicles. Vasopressin stimulation would then induce insertion of p-AQP2 into the apical plasma membrane. Phosphorylation in the PKA site may not necessarily be involved in the last step of regulated trafficking but does induce exocytosis. This mechanism would be consistent with the absence of an overall change in total abundance of p-AQP2 in response to 2 h of DDAVP treatment (both in Brattleboro and normal rats) and the relative high levels of p-AQP2 in vasopressin-deficient Brattleboro rats. The observation that phosphorylation of AQP2 in the PKA consensus site is regulated via V2 receptors may not necessarily undermine the hypothesis, since

![Fig. 5. Immunoblot (A) of membrane fractions from kidney inner medulla of control rats (control) and rats treated with OPC-31260 (1 mg iv) for 30 min (OPC-30') or 60 min (OPC-60') and corresponding densitometric analysis (B). There is a significant reduction in p-AQP2 levels in response to OPC treatment (n = 4, P < 0.005).](http://ajprenal.physiology.org/)

**Fig. 5.** Immunoblot (A) of membrane fractions from kidney inner medulla of control rats (control) and rats treated with OPC-31260 (1 mg iv) for 30 min (OPC-30') or 60 min (OPC-60') and corresponding densitometric analysis (B). There is a significant reduction in p-AQP2 levels in response to OPC treatment (n = 4, P < 0.005).
vasopressin-stimulation/phosphorylation may indeed assure that AQP2 enter a compartment from where it can be recruited for exocytosis.

Vasopressin stimulation may induce phosphorylation of AQP2 residing in intracellular vesicles. This alone or together with other regulatory steps induces exocytosis of p-AQP2 to the apical plasma membrane. This is not immediately consistent with the absence of an overall increase in p-AQP2 levels in response to 2 h of DDAVP treatment. Intuitively, we would have expected p-AQP2 levels to increase in response to DDAVP treatment. There may be several explanations for the absence of the increase. It is likely that there is a temporary increase in p-AQP2 levels in response to acute vasopressin or DDAVP treatment. That vasopressin treatment indeed can induce an increase in p-AQP2 levels was demonstrated in a parallel study using slices of kidney inner medulla. Interestingly, the level of p-AQP2 returned toward baseline in response to continued treatment for 1 h (unpublished results). These observations in in vitro systems are consistent with the present results. Both vasopressin-induced phosphorylation and vasopressin-regulated changes in the osmotic water permeability have been shown to be rapid processes (46, 53). Thus it is possible that levels of p-AQP2 return to the steady state due to dephosphorylation of AQP2 still residing in the apical plasma membrane. Whether this means that phosphorylation of AQP2 is only needed for the trafficking to the membrane but not for AQP2 to reside in the apical plasma membrane remains to be tested in future studies.

The observation that there is a substantial level of phosphorylated AQP2 in intracellular vesicles in untreated normal rats together with the observed marked reduction in p-AQP2 levels in response to vasopressin receptor antagonist treatment supports the view that in collecting duct principal cells of normal untreated rats there is a considerable constitutive activation of PKA (or other kinases) which induces phosphorylation of AQP2 in the vesicular reservoir. AQP1 is structurally organized as homotetramers in the membrane (1), and recent studies indicate that AQP2 also exists as homotramers in the membrane (23). Thus it may be speculated that one or more monomers may be phosphory-

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**Fig. 6.** Immunocytochemistry using thin cryosections (0.85 µm) from a control rat (A) and a rat treated with OPC-31260 for 30 min (B). Sections were labeled with the same concentration of anti-p-AQP2 antibody. In control rats, strong labeling was observed in both plasma membrane domains (arrows) and cytoplasm. A marked reduction in the overall labeling of p-AQP2 in V2-receptor antagonist-treated rats is seen. Magnification, ×1,100.
lated in the unstimulated state (basal state), whereas after vasopressin stimulation more monomers may be phosphorylated resulting in exocytosis. Thus phosphorylation may be required of three or more monomers to induce trafficking.

A third possibility would be vasopressin-regulated trafficking of AQP2 in a nonphosphorylated state. After insertion of nonphosphorylated AQP2 into the apical plasma membrane, AQP2 then becomes phosphorylated by PKA. For this to be the case, it would require that p-AQP2 can be internalized in a phosphorylated state (resulting in the observed p-AQP2 in intracellular vesicles). This hypothesis remains unlikely, mainly because it is inconsistent with the data described above demonstrating that Ser256 is essential for translocation of AQP2 to the membrane (14, 25).

In conclusion, the most likely mechanism involved is vasopressin-induced phosphorylation of AQP2 in an intracellular compartment, and this together with additional steps results in exocytosis of p-AQP2 to the apical plasma membrane. The fraction of p-AQP2 inserted in the membrane is likely to be dephosphorylated, thereby returning total levels of p-AQP2 back to the steady state. The additional steps triggering exocytosis as well as endocytosis of AQP2 remain to be identified.
High Levels of p-AQP2 in Kidneys from Vasopressin-Deficient Brattleboro Rats

There is a high level of p-AQP2 in intracellular vesicles in kidneys from vasopressin-deficient Brattleboro rats. It remains to be identified what stimulates PKA-dependent AQP2 phosphorylation in the absence of vasopressin, but the results suggest an upregulation of other signaling pathways, i.e., PKA may be activated by cAMP through other signal transduction pathways than via V2 receptors. Other ligands using the vasopressin signaling cascade may also be upregulated, e.g., oxytocin, which has been shown to have antidiuretic effects (5). Similar to high levels of p-AQP2, high expression levels of total AQP2 protein have previously been demonstrated in untreated Brattleboro rats (26, 47). There is a cAMP-responsive element in the 5'-flanking region of the AQP2 gene, and several studies have indicated an importance of this in regulating AQP2 gene expression (19, 52, 55). Thus, high activity of cAMP/PKA (or perhaps other kinases) in Brattleboro rat kidney collecting duct might therefore induce both AQP2 protein synthesis and phosphorylation of AQP2 without triggering of the water channel exocytosis.

Regulatory Step(s) Inducing Exocytosis of AQP2

As discussed above, one or more additional steps appear to be necessary in addition to phosphorylation of AQP2 in the PKA consensus site to induce docking and fusion (exocytosis). These factors may include phosphorylation or chemical modification of AQP2 or other proteins participating in the exocytosis.

Other kinases may potentially phosphorylate AQP2 and be involved in regulating AQP2 trafficking (offset or onset response). In addition to the PKA consensus site, AQP2 contains potential phosphorylation sites for protein kinase C (PKC) and casein kinase II (CKII).
These enzymes are both present in the kidney. Several PKC isoforms were identified in rat medullary thick ascending limb and inner medullary collecting duct, and recent studies using renal epithelial cells have demonstrated that PKC is activated by vasopressin (2, 3, 6). CKII activity was observed in bovine kidney and in chicken kidney CKII mRNA has been shown to be expressed (12, 33). cGMP-dependent protein kinase (PKG), Ca$^{2+}$-, calmodulin-dependent protein kinase (PK-CaM) and protein kinase B (PKB) have been shown to phosphorylate the same serine residues as PKA in different proteins (8, 11, 49), and isoforms of these three kinases are expressed in the kidney (16, 39, 50). It is possible that one of these kinases may also phosphorylate Ser$^{256}$ in AQP2.

Phosphorylation of other proteins may also be important for trafficking. Early studies have demonstrated that PKA induces phosphorylation of various mem-

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**Fig. 9.** Electron micrographs of ultrathin Lowicryl HM20 sections from kidney inner medulla of an untreated Brattleboro rat and a DDAVP-treated Brattleboro rat. Sections were immunogold labeled for phosphorylated AQP2. A: in untreated Brattleboro rats, labeling is mainly associated with intracellular vesicles (arrowheads). B: DDAVP treatment for 2 h induces an extensive labeling of the apical plasma membrane (arrows). Labeling is also seen in vesicles (arrowheads). Magnification, $\times$52,300.
brane proteins in bovine kidney (10), and in saponin-permeabilized outer medullary collecting duct segments phosphorylation of at least two unidentified 45- and 66-kDa proteins has been demonstrated (17). Vesicle targeting receptors, also known to be present in the kidney collecting duct, may potentially also be subject to phosphorylation. The vesicle-associated membrane protein VAMP/synaptobrevin and the vesicle-targeting protein SNAP-25 have been shown to be phosphorylated in vitro by protein kinases (18, 40). Furthermore, PKA and CKII can phosphorylate syntaxin-4 in vitro (13), whereas SNAP-23 was not phosphorylated by either PKA or CKII and only minimally by PKC (13). Thus phosphorylation of SNARE proteins may hypothetically play a role for trafficking of AQP2. CKII may also be involved in the regulation of dynein function as evidenced by in vitro studies showing phosphorylation of dynein by CKII as well as binding of CKII to dynein (24).

Regulation of p-AQP2 Levels in Response to V2-Receptor Antagonist Treatment

Phosphorylated AQP2 expression levels were markedly reduced in response to both 30 and 60 min of V2-Receptor antagonist treatment. Previous studies using the same protocol showed no changes in total AQP2 (nonphosphorylated plus phosphorylated) protein abundance after 60 min of OPC-31260 treatment (7). This suggests that V2-Receptor antagonist treatment modulates p-AQP2 abundance at the posttranslational level, i.e., it changes the phosphorylation state of AQP2. V2-Receptor antagonist treatment may either reduce PKA activity and/or induce dephosphorylation of AQP2, e.g., by increased phosphatase activity. Several phosphatases have been identified in the kidney, e.g., protein phosphatases 1 and 2a and the calcium/calmodulin-dependent protein phosphatase calcineurin (4, 31, 51). Whether these are subject to regulation via V2 receptors is unknown. It is likely that there is a constant activity of phosphatases in collecting duct principal cells and that the absence of V2 receptor stimulation (achieved by treatment with V2-receptor antagonist) reduces the buildup of p-AQP2 resulting in a net decrease.

The offset response to vasopressin could be hypothesized to take place by three mechanisms with regard to the role of AQP2 (dephosphorylation). 1) p-AQP2 residing in the apical plasma membrane could be subjected to dephosphorylation, and dephosphorylated AQP2 might then be internalized (either directly as a consequence of dephosphorylation or due to other regulatory steps). 2) Alternatively, phosphorylated AQP2 could be internalized and be subjected to dephosphorylation after internalization. 3) A combination of the two possibilities listed above may exist. This study does not allow us to discriminate between these potential mechanisms.

Conclusion

Our studies strongly support the view that PKA-mediated phosphorylation of AQP2 at Ser256 is involved in vasopressin-regulated trafficking of AQP2 water channel protein. Moreover, the results strongly suggest that other factors are necessary and involved in regulated AQP2 bearing vesicle trafficking, docking, and fusion. These may include further chemical modification of AQP2 and/or involve other regulatory components as discussed above. Future studies will be needed to characterize this in detail.

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Table 2. Semiquantitation of immunogold labeling for phosphorylated AQP2 in the apical part of collecting duct principal cells in Brattleboro rats treated with DDAVP for 2 h

<table>
<thead>
<tr>
<th>Total Labeling Density</th>
<th>APM Linear Labeling Density</th>
<th>APM</th>
<th>VES</th>
<th>NIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>particles/ (\mu m^2)</td>
<td>particles/ nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.3 ± 4.7</td>
<td>60.1 ± 21.1</td>
<td>0.022 ± 0.007</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>DDAVP</td>
<td>39.7 ± 4.8</td>
<td>51.7 ± 55.5</td>
<td>0.21 ± 0.02</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>Total number of gold particles</td>
<td>733</td>
<td>16</td>
<td>16</td>
<td>369</td>
</tr>
<tr>
<td>Control</td>
<td>1,197</td>
<td>255</td>
<td>255</td>
<td>395</td>
</tr>
</tbody>
</table>

Values are means ± SE from 4 Brattleboro rats treated with desmopressin (DDAVP) for 2 h and 3 saline-treated animals. Total labeling density refers to the total density of gold particles per area cytoplasm. Apical plasma membrane (APM) linear labeling density refers to the number of gold particles per length APM. Values in the last 3 columns are expressed as fraction of total labeling of the APM, intracellular vesicles (VES), and nonidentifiable structures (NIS). NIS refers to structures that were not strictly identified as VES or APM (this is likely to represent labeling in tangentially sectioned vesicles or rough endoplasmic reticulum).

Fig. 10. Immunoblot of membrane fractions from kidney inner medulla of control Brattleboro rats (control) and Brattleboro rats treated with DDAVP (50 ng sc) for 2 h. There are no significant changes in p-AQP2 levels after DDAVP treatment.
REFERENCES


