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

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Localization and regulation of PKA-phosphorylated AQP2 in response to V₂-receptor agonist/antagonist treatment. 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-Cys¹,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

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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-methylbenzoyl)amino)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 isoflurane. 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 isoflurane. 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-Cys¹, D-Arg⁸]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 switched to reduce the antigenicity outside the PKA site (peptide 4, Table 1); this antibody has been described previously (46). The peptides were used to immunize rabbits. To remove potential clones recognizing nonphosphorylated AQP2, 1 ml of antiserum was applied three times to a column to which a nonphosphorylated peptide (amino acids 250–271 of rat AQP2 with a cysteine residue at the amino terminus, peptide 1, Table 1) was conjugated. The anti-AQP2 antibody-depleted serum was then applied to a second column containing the phosphorylated AQP2 peptide for affinity purification. The specificity of the affinity-purified anti-p-AQP2 antibodies was ensured by immunoblotting of membrane fractions from kidney inner medulla, the nonphosphorylated peptide, and the phosphorylated peptides.

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 NaO₂, 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-EM embedding prior to ultramicrotomy. Cryosubstitution was performed as described previously (37, 45). The frozen samples were freeze-substituted in a Reichert AFS (Reichert, Vienna, Austria). Samples were sequentially equilibrated over 3 days.
in 0.5% uranyl acetate in methanol at temperatures gradually increasing from −80°C to −70°C, then rinsed in pure methanol for 24 h at −70°C to −45°C, and infiltrated at −45°C with Lowicryl HM20 and methanol 1:1, 2:1, and finally pure Lowicryl HM20 (1 day in each solution) before ultraviolet polymerization in pure Lowicryl HM20 for 2 days at −45°C and 2 days at 0°C.

Semithin (0.05 µm) cryosections and ultrathin (80 nm) Lowicryl sections, cut on a Reichert Ultracut FCS, were preincubated with PBS containing 0.1% skimmed milk and 0.05 M glycine (for light microscopy) or with TBST (0.05 M Tris, pH 7.4, 0.1% Triton X-100) containing 0.1% sodium borohydride and 0.05 M glycine followed by incubation with TBST containing 0.2% skimmed milk (for electron microscopy). The preincubation was followed by incubation with affinity-purified anti-p-AQP2 or anti-AQP2 antibody. For light microscopy, labeling was visualized by use of peroxidase affinity-purified anti-p-AQP2 or anti-AQP2 antibody. For electron microscopy, labeling was visualized by use of colloidal gold particles (GAR.EM10 or GAFR.EM10, 1:50; Biocell Research Laboratories, Cardiff, UK), and grids were stained with uranyl acetate for 10 min and with lead citrate for 30 s.

Semi-Quantitation Of Phosphorylated AQP2 Immunogold Labeling

Electron micrographs were taken of the apical part of inner medullary collecting duct principal cells from Brattleboro rats treated with DDAVP (sc) for 2 h (n = 4) and control Brattleboro rats receiving saline treatment (sc) for 2 h (n = 3).

Fig. 1. Immunoblot of membrane fractions from kidney inner medulla (lane 5), nonphosphorylated AQP2 peptide (AQP2 peptide; peptide 1; 2 µg, lanes 1 and 2; and 1 µg, lanes 3 and 4) and phosphorylated AQP2 peptide (p-AQP2 peptide, peptide 2; 1 µg, lanes 6 and 7 and 2 µg, lanes 8 and 9). The anti-AQP2 antibody recognizes both peptides and in kidney membranes the antibody recognizes 29-kDa and 35- to 50-kDa bands, corresponding to nonglycosylated AQP2 and glycosylated forms of AQP2 (lane 5). There are no significant differences in the affinity for the nonphosphorylated and the phosphorylated peptides, IM, inner medulla.
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 intracellular vesicles in collecting duct principal cells.

Effects of V$_2$-Receptor Antagonist Treatment on p-AQP2 Levels and Intracellular Distribution in Normal Rats

To assess the effect of V$_2$-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 V$_2$-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-

![Fig. 3. Thin cryosections (0.85 µm) of kidney inner stripe outer medulla (A and B) and inner medulla (C and D) of normal rats. Sections were incubated with affinity-purified anti-AQP2 (A) or affinity-purified anti-p-AQP2 antibody (B–D), and labeling was visualized using peroxidase-conjugated secondary antibody. A: anti-AQP2 antibody labels apical plasma membrane domains (arrows) and intracellular domains in collecting duct principal cells. There is no labeling of intercalated cells (arrowheads). B: p-AQP2 labeling is associated with apical plasma membrane domains (arrows) in collecting duct principal cells in inner stripe outer medulla. Inset: labeling of intracellular domains (arrowheads) when using a higher concentration of antibody. C: in inner medulla anti-p-AQP2 antibody labels apical plasma domains (arrows). D: labeling with a higher concentration of the antibody shows presence of p-AQP2 in intracellular domains (arrowheads) and plasma membrane domains (arrows). Absorption controls using preabsorbed anti-p-AQP2 as primary antibody reveals no labeling (inset, C). Magnification, ×860.

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-

Fig. 4. Electron micrograph of an ultra-thin Lowicryl HM20 section from kidney inner medulla of a normal untreated rat. Section was immunogold labeled for p-AQP2. p-AQP2 labeling is seen in apical plasma membrane (arrows) and in intracellular vesicles (arrowheads). Magnification, $\times 63,000$. 
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 targeting/docking of AQP2 bearing vesicles. It is likely that phosphorylation in this site plays a permissive or facilitatory role in trafficking but does not itself 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
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 homotetramers 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 Ser^{256} 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.

Fig. 7. Immunoelectron microscopy of p-AQP2 in ultrathin Lowicryl sections from kidney inner medulla of normal rats treated for 30 min with saline (A) or OPC-31260 (B). Only very sparse labeling of intracellular vesicles is seen in OPC-31260-treated rats (arrowheads) compared with saline-treated rats. Magnification, ×60,000.
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).

Fig. 8. Thin cryosections (0.85 µm) of kidney inner medulla of an untreated Brattleboro rat (A) and a Brattleboro rat treated with [desamino-Cys¹,D-Arg⁸]vasopressin (DDAVP) for 2 h (B). Sections were incubated with affinity-purified anti-p-AQP2 antibody and peroxidase-conjugated secondary antibody. A: distinct phosphorylated AQP2 labeling of intracellular domains is seen in untreated Brattleboro rats (arrowheads), whereas only faint labeling of the apical plasma membrane is observed (arrows). B: 2 h of DDAVP treatment increases markedly the labeling of apical plasma membrane domains (arrows). Magnification, ×1,100.
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 Ser256 in AQP2.

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

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, ×52,300.
bran 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 subjected 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 V_2-Receptor Antagonist Treatment

Phosphorylated AQP2 expression levels were markedly reduced in response to both 30 and 60 min of V_2-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 V_2-receptor antagonist treatment modulates p-AQP2 abundance at the posttranslational level, i.e., it changes the phosphorylation state of AQP2. V_2-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 V_2 receptors is unknown. It is likely that there is a constant activity of phosphatases in collecting duct principal cells and that the absence of V_2 receptor stimulation (achieved by treatment with V_2-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 (de)phosphorylation. 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.

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

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<th>APM Linear Labeling Density</th>
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<td>Control</td>
<td>40.3 ± 4.7</td>
<td>0.022 ± 0.007</td>
<td>0.50 ± 0.07</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td>DDAVP</td>
<td>39.7 ± 4.8</td>
<td>0.21 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>0.46 ± 0.05</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).

Conclusion

Our studies strongly support the view that PKA-mediated phosphorylation of AQP2 at Ser \(^{256}\) 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 modifications 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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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


