Phosphorylation of Aquaporin-2 Does Not Alter the Membrane Water Permeability of Rat Papillary Water Channel-containing Vesicles*

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Antidiuretic hormone modulates the water permeability (Pf) of epithelial cells in the rat kidney by vesicle-mediated insertion and removal of the aquaporin-2 (AQP-2) channel. AQP-2 possesses a single consensus CAMP-dependent protein kinase A (PKA) phosphorylation site (Ser-256) hypothesized to regulate channel Pf (Kuwahara, M., Fushimi, K., Terada, Y., Bai, L., Sasaki, S., and Marumo, F. (1995) J. Biol. Chem. 270, 10384–10387). To test whether PKA phosphorylation of AQP-2 alters channel Pf, we compared the Pf values of purified AQP-2 endosomes after incubation with either PKA or alkaline phosphatase. Studies using [γ-32P]ATP reveal that AQP-2 endosomes contain endogenous PKA and phosphatase activities that add and remove 32P label from AQP-2. However, the Pf (0.16 ± 0.06 cm/s) of endosomes containing phosphorylated AQP-2 (0.7 ± 0.3 mol of PO4/mol of protein) is not significantly different from the same AQP-2 endosomes where 95 % of the phosphate has been removed (Pf, 0.14 ± 0.06 cm/s). These data do not support a role for PKA phosphorylation in alteration of AQP-2's Pf. Instead, AQP-2 phosphorylation by PKA may modulate AQP-2's distribution between plasma membrane and intracellular vesicle compartments.

ADH stimulation of rat kidney IMCD causes a rapid increase in its apical membrane osmotic water permeability (Pf) (1-3). This large ADH-elicted increase in Pf occurs by the fusion of cytoplasmic vesicles containing water channels with the apical membrane (1, 4, 5). Withdrawal of ADH stimulation induces retrieval of apical membrane water channels by endocytosis and returns apical membrane Pf to its low base-line value (6-8). In the rat kidney IMCD, this insertion and removal process is mediated by an increase in intracellular cAMP and activation of cAMP-dependent PKA (9, 10).

Data from many laboratories have now established the central role of aquaporin water channel proteins as water-selective pores in the plasma membranes of various renal epithelial cells (reviewed in Refs. 2, 3, and 11). Reconstitution studies of AQP-1 in liposomes suggest that AQP water channel proteins exist as homotetramers in plasma membranes where each AQP polypeptide forms a narrow water-selective channel (12). A total of four AQPs expressed in the mammalian kidney have been cloned and characterized. All four AQPs possess six transmembrane domains composed of highly conserved sequences, while the structures of their carboxyl-terminal regions are divergent (2, 3). Antiserum specific for the respective carboxyl-terminal sequences of various AQPs have been utilized to localize these proteins to either the apical or basolateral plasma membranes and/or vesicles within individual cell types within specific nephron segments (13-16).

A large body of data demonstrates that AQP-2 is the ADH-elicted water channel. AQP-2 is expressed exclusively by ADH-responsive cells in rat collecting duct (14, 17, 18), where it is prominently located in the apical membrane as well as a population of subapical vesicles (13, 19). Recent ultrastructural studies have demonstrated that ADH stimulation and withdrawal redistributes AQP-2 from cytoplasmic vesicles to the apical membrane followed by retrieval into endosomes (19-21). Purified endosomes originating from the apical membrane of rat IMCD are highly enriched for AQP-2 (22). Finally, structural alterations of the human AQP-2 gene are associated with the disease nephrogenic diabetes insipidus, where affected individuals lack the ability to produce hypertonic urine despite high serum ADH levels (23).

The cDNA sequence of AQP-2 predicts a 271-amino acid protein with one N-linked glycosylation site (17, 18). This corresponds to a 29-kDa protein and its 35-40-kDa glycosylated form as identified by multiple anti-AQP-2 antisera (13, 18). In addition, the cDNA sequence corresponding to the carboxyl-terminal domain of AQP-2 reveals several putative phosphorylation sites including a cAMP-dependent protein kinase A (PKA) site (Ser-256), a site for protein kinase C (Ser-226), and two potential sites of casein kinase phosphorylation (Ser-229 and Thr-244). (17). The presence of these potential phosphorylation sites has raised the possibility that the Pf of individual AQP-2s may be altered by phosphorylation in a manner similar to that described for other ion channels (24-27). This includes alteration of channel function by PKA phosphorylation (28) of the major intrinsic protein of the lens (29) that possesses a 59% sequence homology with AQP-2 (30).

Expression studies of AQP-2 in Xenopus oocytes demonstrate that preincubation with cAMP or its analogs increases the Pf of
oocytes injected with either total medullary RNA (31) or cRNA from wild type AQP-2 (17, 32). In recent studies, Kuwahara and co-workers (32) have utilized site-directed mutagenesis techniques in an attempt to identify a potential role for cAMP-mediated PKA phosphorylation of Ser-256 in modulation of AQP-2 function. Their data demonstrate that alteration of AQP-2 Ser-256 results in a loss of the 2-fold increase in P$_f$ induced by preincubation of oocytes expressing AQP-2 with cAMP. To distinguish between the cAMP-mediated increases in either 1) the P$_f$ of individual AQP-2 proteins resident in the oocyte plasma membrane or 2) alterations in the number of AQP-2 proteins present in the oocyte membrane by insertion and retrieval of AQP-2-containing membrane, these studies quantified binding of an anti-AQP-2 antiserum to the external surface of the oocyte membrane. Since no differences in anti-AQP-2 binding to oocytes were observed after cAMP stimulation, these authors suggested that cAMP-mediated phosphorylation of Ser-256 alters the P$_f$ of individual AQP-2 proteins.

To further test the conclusions from these data, we have utilized a homogeneous population of purified endosomes derived from the apical membrane of rat IMCD that are highly enriched for AQP-2 (22). We tested whether AQP-2 is phosphorylated or dephosphorylated by endogenous membrane-bound enzymes present in these purified endosomes and if phosphorylation of AQP-2 alters endosomal membrane P$_f$. Our data show that purified IMCD endosomes possess endogenous PKA and phosphatase activities that phosphorylate and dephosphorylate AQP-2. However, paired measurements of membrane P$_f$ show no significant differences between endosomes containing phosphorylated AQP-2 after incubation with exogenous PKA as compared to the same endosomes where AQP-2 was dephosphorylated after incubation with exogenous PKA as compared to the same endosomes where AQP-2 is dephosphorylated. However, paired measurements of membrane P$_f$ show no significant differences between endosomes containing phosphorylated AQP-2 after incubation with exogenous PKA as compared to the same endosomes where AQP-2 is dephosphorylated by alkaline phosphatase treatment. These data do not support a role for cAMP-mediated PKA phosphorylation in regulating the permeability of the AQP-2 water channel.

**EXPERIMENTAL PROCEDURES**

Materials—Female Sprague-Dawley rats (200–250 g) were purchased from Charles River Laboratories (Cambridge, MA). Tris was obtained from U.S. Biochemical Corp.; glycine was from J.T. Baker Inc.; SDS, acrylamide, ammonium persulfate, and 2-mercaptoethanol were from Bio-Rad. All other chemicals were from Sigma. Anti-fluorescein and anti-AQP-2 antibodies were purchased as described previously (14, 22).

Isolation of AQP-2 Endosomes—Endosomes were prepared from the inner medulla and papilla of rats as described previously (22). In experiments using stopped-flow fluorimetry, endosomes were loaded with F-dextran (10 kDa) after intravenous injection of rats with 250 mg/kg of purified AQP-2 in 0.1% Amido Black. Equal portions of the same 32P-labeled AQP-2 band were incubated with or without 2-mercaptoethanol released denatured 32P-labeled AQP-2 without quantitative denaturation of light and heavy IgG chains of anti-AQP-2 antibody. Thus, subsequent SDS-PAGE purification of the AQP-2 35–45-kDa band was performed localized to the 35–40-kDa region of the 35–45-kDa protein band that is completely free of contaminating IgG as determined by a combination of protein staining and ECL blotting using affinity-purified anti-rabbit antiserum as described above (data not shown).

Determination of the Phosphate Content of Purified 32P-Labeled AQP-2 Protein—After phosphorylation of endosomes for 3 min using exogenous PKA as described above, purified 32P-labeled AQP-2 protein was isolated by immunoprecipitation and SDS-PAGE. Incubation of endosomes with AKAP S10 (Sigma) and released denatured 32P-labeled AQP-2 without quantitative denaturation of light and heavy IgG chains of anti-AQP-2 antibody. Thus, subsequent SDS-PAGE purification of the AQP-2 35–45-kDa band was performed localized to the 35–40-kDa region of the 35–45-kDa protein band that is completely free of contaminating IgG as determined by a combination of protein staining and ECL blotting using affinity-purified anti-rabbit antiserum as described above (17).

Determination of the Osmotic Water Permeability (P$_w$) of AQP-2 Endosomes—Paired measurements of endosomal water flux (J$_w$) were performed to compare the P$_w$ values of endosomes containing AQP-2 in either a maximally phosphorylated or dephosphorylated state. AQP-2 endosomes were first phosphorylated for 3 min as described above, and the J$_w$ of one half of the single preparation determined immediately. AQP-2 in the remaining half of the endosomes was then dephosphorylated using alkaline phosphatase as described above and the J$_w$ of the aliquot determined. J$_w$ was measured as described previously using a SF.17MV stopped-flow fluorimeter (Applied Photophysics, Leatherhead, United Kingdom) with a measured dead time of 0.7 ms configured in the fluorescence mode (22, 36). Endosomal shrinkage due to water efflux was monitored as a function of the self-quenching of F-dextran entrapped within the endosomal lumen. Stopped-flow fluorimetry data were collected, averaged, and fitted to single exponential curves (Applied Photophysics) and P$_w$ determined as described previously (12, 22, 36).

To insure that phosphorylation did not alter the mean diameters of AQP-2 endosomes, paired samples were fixed and sectioned and mean diameters of endosomes visualized under identical magnifications were determined as described previously (22).

**RESULTS**

In previous work, we have employed a protocol consisting of five separate differential centrifugation steps combined with Percoll gradient sedimentation to purify a homogeneous population of apically derived endosomes from homogenates of rat kidney inner medulla and papilla (22). These endosomes average 144 ± 5 nm in diameter, contain functional water channels, and are highly enriched for AQP-2 protein as determined by immunoblotting using a specific rabbit anti-AQP-2 antiserum (14, 22). In experiments described below, these AQP-2 endosomes utilized to determine the functional consequences.
AQP-2 Sepharose 4B-linked antiserum (lanes 2-6), Mg$_2^+$ of AQP-2 phosphorylation by PKA. $^{32}$P-Labeled proteins were identified by SDS-PAGE and autoradiography after solubilization of individual aliquots either in SDS directly (lanes 1, 3, 5, and 7) or after immunoprecipitation of AQP-2 by anti-AQP-2 Sepharose 4B-linked antiserum (lanes 2, 4, 6, and 8). Incubation conditions included: 0.1 mM $[^{32}$P$]ATP$ only (lanes 1 and 2), in combination with (20 units/ml) PKA (lanes 3 and 4), together with either the regulatory subunit of PKA (lanes 5 and 6) or a 20-mer peptide IP$_{30}$ (35) to the regulatory subunit of PKA phosphorylation (lanes 7 and 8). The representative autoradiogram was developed after 24 h and is representative of eight separate experiments. The position of proteins of known molecular mass $\times 10^{-3}$ are indicated by the arrowheads, while the top (T) and dye front (D) of the gel are shown by the small arrowheads. In lanes 1 and 3, proteins $^{32}$P-labeled by addition of ATP only are indicated by small arrowheads or brackets. Stars indicate bands corresponding to the 28- and 35–45-kDa AQP-2 bands shown in lane 4 as well as a fainter band of 55 kDa corresponding to the regulatory subunit of PKA as shown in lane 5 but not lane 6.

of AQP-2 phosphorylation by PKA.

As shown in Fig. 1, incubation of AQP-2 endosomes with Mg$^{2+}$-$[^{32}$P$]ATP$ alone (lane 1) results in the appearance of 7 major $^{32}$P-labeled SDS-PAGE protein bands of approximately 100–150, 94, 40–43, 30–34, and 22–24 kDa as well as a band of very large molecular mass greater than 200 kDa. Their respective locations in autoradiograms of total endosomal proteins shown in Fig. 1 (lanes 1 and 3) are indicated by small arrowheads or brackets. However, Triton X-100 solubilization and immunoprecipitation of the endosomal proteins shown in lane 1 with anti-AQP-2 Sepharose shows AQP-2 protein is not phosphorylated (Fig. 1, lane 2). In contrast, addition of purified catalytic PKA subunit to these endosomes results in an overall increase in $^{32}$P labeling of endosomal protein bands (Fig. 1, lane 3) and prominent $^{32}$P labeling of AQP-2 protein in anti-AQP-2 immunoprecipitates (Fig. 1, lane 4). This includes protein bands of 28 and 35–45 kDa corresponding to the nonglycosylated and glycosylated forms of AQP-2 as described previously (18). The locations of AQP-2 bands present in autoradiograms of whole endosomal proteins are indicated by stars of Fig. 1 (lane 3). Under these conditions, phosphorylation of AQP-2 could not be attributed directly to PKA since $^{32}$P labeling of AQP-2 was not observed after addition of either an excess of purified PKA regulatory subunit (lanes 5 and 6) or IP$_{30}$, a 20-mer peptide that is a highly specific competitive inhibitor of PKA catalytic activity (Fig. 1, lanes 7 and 8) (35). As expected, addition of excess regulatory subunit (molecular mass 55 kDa) to endosomes results in the appearance of a prominent 55-kDa $^{32}$P-labeled band shown in lane 5 that is not present in lane 7. A star in lane 3 denotes the location of a fainter 55-kDa band that appears upon addition of both $[^{32}$P$]ATP$ and PKA catalytic subunit that likely represents phosphorylation of endogenous AQP-2 regulatory subunit by exogenous PKA.

The ratio of $^{32}$P labeling between the 35–45- and 28-kDa AQP-2 bands after 5 min of $[^{32}$P$]ATP$ incorporation by PKA and purification by immunoprecipitation was 3.75 $\pm$ 0.57 (n = 12) as determined by densitometry of autoradiograms of anti-AQP-2 immunoprecipitates. This value is comparable to the ratio of these AQP-2 protein bands as detectable by immunoblotting (14, 18, 22). These data demonstrate that under conditions described in Fig. 1, both the 28- and 35–45-kDa forms of AQP-2 present in these purified endosomes are specific substrates for PKA phosphorylation.

Previous work in brain (reviewed in Refs. 37 and 38), erythrocytes (39), epithelial (40), and neuroendocrine cells (41) has demonstrated that hormone activation causes cAMP accumulation in distinct cellular compartments where PKA subunits are differentially localized and often bound to membranes through their association with a family of PKA-anchoring proteins. In the kidney medulla and papilla, a significant portion of total cAMP-dependent PKA activity is present in the particulate fractions of homogenates (42). To determine if bound endogenous cAMP-dependent PKA activity is present in endosomes where it could phosphorylate AQP-2, purified endosomes were incubated in the presence of 100 $\mu$M cAMP and $[^{32}$P$]ATP$ only (Fig. 2, lanes 1 and 4). The patterns of phosphorylation obtained in SDS-PAGE analysis of both whole endosomal proteins (lane 1) and anti-AQP-2 immunoprecipitates (lane 4) were then compared to their respective counterparts after incubation of AQP-2 endosomes with either $[^{32}$P$]ATP$ alone (Fig. 2, lanes 2 and 5) or after addition of a combination of $[^{32}$P$]ATP$ and exogenous PKA catalytic subunit (Fig. 2, lanes 3 and 6). Addition of 100 $\mu$M cAMP consistently resulted in $^{32}$P labeling of AQP-2 (lane 4) that was similar to that present after addition of both $[^{32}$P$]ATP$ and exogenous PKA (lane 6). $^{32}$P labeling of AQP-2 was absent after incubation of
endosomes with \([\gamma-^{32}P]\)ATP only (lane 5). These data demonstrate the presence of endogenous PKA activity that is activated by 100 \(\mu\)M cAMP and phosphorylates AQP-2 protein in these endosomes.

To obtain conditions where maximal phosphorylation of AQP-2 was achieved after addition of exogenous PKA catalytic subunit, the time course of \(^{32}P\) labeling of AQP-2 was determined as shown in Fig. 3A. Maximal incorporation of \(^{32}P\) into AQP-2 occurred within 3 min (Fig. 3A, lanes 2 and 6) and was followed by progressive loss of label over an interval of 20 min (Fig. 3A, lanes 3, 4, 7, and 8). Quantitation of autoradiograms of immunoprecipitates using anti-AQP-2 antiserum (lanes 1–4) showed loss of 47 ± 23% (\(n = 4\)) of AQP-2 \(^{32}P\) label after 10 min as compared to that present after 3 min of phosphorylation by PKA. After 20 min, only 25 ± 13% (\(n = 4\)) of \(^{32}P\) label remained in AQP-2. There were no significant differences observed in ratios of \(^{32}P\) content of 45–35-kDa/28-kDa AQP-2 bands at 3 min (3.4 ± 0.43; \(n = 4\)), 10 min (3.71 ± 0.29; \(n = 4\)), or 20 min (3.82 ± 0.30; \(n = 4\)).

The progressive reduction of \(^{32}P\)-labeled AQP-2 present in immunoprecipitates shown in Fig. 3A could result from either dephosphorylation by endogenous phosphatase activity or dephosphorylation of AQP-2 during the interval that endosome proteins are phosphorylated. To distinguish between these possibilities, identical aliquots of endosomes were either preincubated under phosphorylation conditions for 20 min at 37°C (Fig. 3B, lane 1) or held on ice (Fig. 3B, lane 2). After subsequent phosphorylation of endosomes for 5 min, the content of \(^{32}P\)-labeled immunoprecipitable AQP-2 in each aliquot was compared by autoradiography. The \(^{32}P\)-labeled AQP-2 content of endosomes preincubated under phosphorylation conditions (panel B, lane 1) was not significantly different (0.98 ± 0.05; \(n = 3\)) as compared to control samples (panel B, lane 2). These data suggest that the progressive loss of \(^{32}P\) label from AQP-2 in purified endosomes results from endogenous phosphatase activity. Under these conditions, neither the 35–45-kDa nor the 28-kDa AQP-2 band appears to be present for the following substrates for dephosphorylation.

As shown in Fig. 4, incubation of endosomes with 150 \(\mu\)g/ml alkaline phosphatase for 20 min at 37°C resulted in a loss of greater than 95 ± 8% (\(n = 7\)) of \(^{32}P\) label from purified immunoprecipitates of AQP-2. Prior to quantitation of the \(^{32}P\)-labeled phosphate content of AQP-2, the \(^{32}P\) label resulted from AQP-2 in endosomes subjected to preincubation with alkaline phosphatase was compared to that displayed by control endosomes. This experiment was performed to determine if AQP-2 present in endosomes already possesses a significant content of endogenous nonradioactive phosphate prior to its phosphorylation in vitro by exogenous PKA and \([\gamma-^{32}P]\)ATP. Preincubation with alkaline phosphatase would remove any endogenous phosphate and thus be expected to increase incorporation of \(^{32}P\) label into AQP-2 in subsequent exogenous PKA phosphorylation. However, as shown in Fig. 5, paired experiments revealed no significant difference (0.91 ± 0.1; \(p > 0.05\); \(n = 4\)) in the \(^{32}P\) label of AQP-2 derived from either control (lane 2) or alkaline phosphatase-treated (lane 1) endosomes. These data suggest that AQP-2 present in purified endosomes does not possess a significant content of phosphate prior to its phosphorylation with exogenous PKA.

To quantify the \(^{32}P\) phosphate content of AQP-2, \(^{32}P\)-labeled AQP-2 protein was purified by immunoprecipitation and SDS-PAGE. As described under “Experimental Procedures,” the 35-
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FIG. 5. Preincubation of AQP-2 endosomes with alkaline phosphatase does not alter its content of 32P label after subsequent phosphorylation with exogenous PKA. Identical aliquots of AQP-2 endosomes were preincubated in the absence (Cont) or presence (Alk.) of alkaline phosphatase as described in Fig. 4. AQP-2 endosomes were then repeatedly centrifuged in ice-cold Buffer B to remove soluble alkaline phosphatase enzyme. Both endosome aliquots were then phosphorylated and analyzed as described for lane 4 of Fig. 1. The autoradiogram exposed for 36 h displays a representative experiment performed a total of four times. Arrows located on the right denote the 28- and 35–45-kDa 32P-labeled AQP-2 bands.

40-kDa region of individual 35–45-kDa AQP-2 protein bands was used for analyses since prior studies had demonstrated that it was free of contamination by IgG protein present in AQP-2 immunoprecipitates (data not shown). Quantitation of 32P content by scintillation counting and protein by amino acid analysis showed AQP-2 protein possessed 0.7 ± 0.3 (n = 3) mol of 32P phosphate/mol of AQP-2 protein.

Having established conditions where AQP-2 in endosomes contains an average of 0.7 mol of phosphate/mol of protein (Fig. 5) or greater than 95 ± 8% of the 32P phosphate is removed by alkaline phosphatase (Fig. 4), the membrane water flux (Jv) of these respective endosomes was compared in a series of paired experiments. AQP-2 endosomes containing entrapped F-dextran were prepared from rats receiving intravenous F-dextran injections and the respective endosomes was compared in a series of paired experiments. AQP-2 endosomes containing entrapped F-dextran were prepared from rats receiving intravenous F-dextran injections and the Jv of aliquots of individual preparation of endosomes determined using stopped-flow fluorimetry (22, 36). There was no significant difference between the magnitude of entrapped fluorescence as well as the Jv of endosomes phosphorylated by PKA and ATP (Fig. 6, upper panel) as compared to those displayed when these endosomes were subjected to dephosphorylation by alkaline phosphatase treatment (Fig. 6, lower panel). Ultrastructural analyses also revealed no significant difference (p > 0.5) in the mean diameter of phosphorylated AQP-2 endosomes (144 ± 15 nm; n = 50) as compared to dephosphorylated endosomes (146 ± 9 nm; n = 50). Hence, the P50 of endosomes containing phosphorylated AQP-2 (0.16 ± 0.06 cm/s; n = 3) was not significantly different from the P50 of endosomes where AQP-2 had been dephosphorylated by alkaline phosphatase (0.14 ± 0.06 cm/s; n = 3). These data demonstrate that alterations in the phosphorylation state of AQP-2 do not result in significant changes in the P50 of these endosomes.

DISCUSSION

The data contained in Figs. 1 and 2 demonstrate that AQP-2 protein present in purified endosomes can be phosphorylated by both endogenous PKA or exogenous PKA catalytic subunit in vitro. Although addition of only [γ-32P]ATP to endosomes results in phosphorylation of several proteins, both the 28- and 35–45-kDa bands of AQP-2 are phosphorylated exclusively after addition of cAMP or exogenous PKA catalytic subunit. Thus, under these in vitro conditions, phosphorylation of AQP-2 by other kinase activities does not occur.

Kinetic studies of 32P incorporation into AQP-2 reveals its rapid phosphorylation by PKA followed by a net loss of 32P label (Fig. 3, panel A). Present data suggest that the loss of 32P label from AQP-2 results from endogenous phosphatase activity rather than proteolysis. First, preincubation of endosomes under these conditions prior to AQP-2 phosphorylation results in no detectable loss of immunoprecipitable 32P-labeled AQP-2 as compared to control (Fig. 3, panel B). Had significant proteolysis of AQP-2 occurred during the preincubation interval, we would have anticipated a reduction in the 32P-content of AQP-2 as compared to control. Second, our anti-AQP-2 antiserum specifically recognizes the amino acid sequence (residues 258–271) located immediately adjacent to the consensus PKA phosphorylation site at Ser-256 of AQP-2 (14). If limited proteolysis of AQP-2 had occurred, then additional 32P-labeled AQP-2 bands would likely be present in autoradiograms of AQP-2 immunoprecipitates. However, we have observed no such additional 32P bands in our AQP-2 immunoprecipitates.

The presence of bound PKA and phosphatase activities in purified AQP-2 endosomes is intriguing because recent reports have demonstrated a specific role for both membrane-bound PKA (43) and phosphatase enzymes (44) in modulation of func-
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