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Protein Kinase A-Dependent Phosphorylation of Aquaporin-1

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The molecular mechanisms for regulating water balance in many tissues are unknown. Like the kidney, the eye contains multiple water channel proteins (aquaporins) that transport water through membranes, including two (AQP1 and AQP4) in the ciliary body, the site of aqueous humor production. Previous results from our laboratory demonstrated that water channel activity of AQP1 was significantly increased by protein kinase A (PKA) activators such as cyclic-AMP (cAMP) and forskolin. The purpose of this study is to determine whether PKA-dependent protein phosphorylation is involved in the regulation of water channel activity of AQP1. Results presented here suggest that catalytic subunit of protein kinase A significantly increased the amount of phosphorylated AQP1 protein. In addition, these results indicated that cAMP-responsive redistribution of AQP1 may be regulated by phosphorylation of AQP1. Moreover, they provide new insights on the molecular mechanisms for regulating water balance in several tissues involving rapid water transport such as ciliary epithelium. In addition, they suggest important potential roles for AQP1 in several clinical disorders involving rapid water transport such as glaucoma.

Key Words: **aquaporins; phosphorylation; fluid transport.**

The recent discovery of "aquaporins," a large family of membrane proteins that function as highly selective water channels (reviewed in Refs. 1–7), has drawn attention to their role in physiology and in several human diseases that involve rapid water transport, and have identified them as potential targets for therapeutic intervention. At least 10 aquaporins (AQPs), numbered 0 through 9, have been identified from various mammalian tissues. The finding of homologous genes in amphibians, insects, and bacteria highlights the evolutionary conservation and thus probable importance of these proteins. These proteins are similar in size (256–281 amino acids) and a single polypeptide chain spans the membrane six time. The two halves of the molecule exhibit substantial sequence similarity to one another but are oriented oppositely in the membrane so that corresponding regions are found on opposite sides of the membrane and contain the Asp-Pro-Ala (NPA) sequence that is characteristic of the major intrinsic protein of the lens (MIP) family of proteins of which the AQPs are members. AQP1, AQP2, AQP3, and AQP7 have been shown to transport nonionic small solutes such as urea and glycerol in addition to water (8–11), whereas AQP4, AQP5, AQP6, and AQP8 are highly selective to water permeation and exclude small solutes. A recent report indicates that AQP1 channels have the capacity to participate in ionic signaling after the activation of cGMP second messenger pathways (12). Although structurally similar to the AQPs other non-AQP MIP analogs have not been demonstrated to transport water.

Recent studies have identified the specific roles for aquaporins in well recognized physiologic processes and diverse pathophysiologic disorders common in the practice of clinical medicine. For example, mutations in AQP0 (MIP26, moderate water conductance) result in congenital cataracts (13); and mutations in AQP2 (vasopressin-regulated kidney water channel) result in a severe form of nephrogenic diabetes insipidus (14, 15). In addition, knockout mice studies with selective deletions of various aquaporins suggests a role for AQP1 in kidney and lung fluid transport and AQP4 in colonic fluid transport and brain edema (16–23).

Several investigators including our laboratory have identified the presence of aquaporins in the eye (24–26). At present five different aquaporins (AQP0, AQP1, AQP3, AQP4, AQP5) are expressed in the eye, and these may provide a physiologic basis for water and fluid movement in selective tissues in the eye. Previous

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studies using immunocytochemistry, RT-PCR and northern blotting confirmed the expression of AQP1 and AQP4 in the nonpigmented layer of ciliary body (25, 26), a major site for the aqueous humor production. We recently demonstrated the regulation of AQP1 by cAMP, forskolin and vasopressin using oocyte swelling assay. Since the application of forskolin to the eye reduce intraocular pressure (IOP) (27), we postulated that cAMP-dependent regulation of AQP1 water channel activity may account for the observed reduction of IOP using these agents. We demonstrate here that water channel activity of AQP1 is regulated by cAMP-dependent protein phosphorylation by PKA. In addition, our results indicate that phosphorylation of AQP1 may be involved in cAMP responsive redistribution of AQP1.

METHODS

In vitro cRNA synthesis of AQP1. The *Eco*R1–*Bam*H1 fragment of bovine AQP1 (CHIP29) containing 32 bp of the 5′-untranslated sequence, 205 bp of the 3′-untranslated sequence, and the entire aqp1 open reading frame (28) was blunt-ligated into the *Bgl*II site of the *Xenopus* expression construct pXbG as described previously (29). Conventional molecular biology techniques along with commercially available restriction endonucleases were used to identify the orientation of the cDNA into the recombinant plasmid. Confirmation of the recombinant plasmid was made by nucleotide sequencing. Sense and antisense capped RNA transcripts of AQP1 were synthesized *in vitro* with T3 RNA polymerase using two recombinant plasmids with the AQP1 cDNA cloned in sense and antisense direction.

Oocytes membrane isolation and immunoblot analysis. Defolliculated stage V and VI oocytes from female *Xenopus laevis* (30) were injected with 20 nl of water or cRNAs (1 mg/ml). After incubation in 200 mOsm modified Barth's buffer at 18°C for 72 h, oocytes were treated with 8-bromo-cAMP (a membrane permeable analog of cAMP) and arginine-vasopressin (AVP) in Barth's buffer containing appropriate concentrations of the reagent for 30 min prior to membrane preparations. Groups of 8-10 oocytes were transferred with Barth's buffer into 1.5-ml microcentrifuge tubes on ice. After chilling for 10 min, the Barth's buffer was replaced by 0.5–1 ml of ice-cold lysis buffer (10 mM Na_2HPO_4, pH 7.4, containing 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) and oocytes were lysed by vortexing and repetitive pipetting the samples. The yolk and cellular debris were removed by centrifuging the lysates at 1000*g* × 5 min at 4°C. The membranes were collected after centrifugation of the supernatant at 20,000*g* for 30 min at 4°C. The membranes were carefully washed once with the ice cold lysis buffer and were resuspended in 20 μl SDS–polyacrylamide gel electrophoresis (PAGE) sample loading buffer containing 2% SDS and electrophoresed into a 12% SDS–PAGE, transferred to nitrocellulose, incubated with a 1:1250 dilution of affinity purified anti-AQP1 antibody (Alomone Labs, Jerusalem, Israel), and the immunoreactive bands were visualized using an ECL Western blotting detection system (Amersham).

N-Glycosidase F digestions. Deglycosylation of membrane proteins from *Xenopus* oocytes were performed in 50 μl of incubation buffer (20 mM sodium phosphate, 10 mM EDTA, 1% 2-mercaptoethanol, 1% SDS, pH 7.0). The membranes were heated for 10 min at 80°C. SDS was neutralized by the addition of 25 μl of 20% *n*-dodecyl maltoside, and supernatant was incubated at 37°C with 2 units of *N*-glycosidase F (Boehringer Mannheim) overnight. Deglycosylated proteins were precipitated with methanol/chloroform as described. (31)

In vitro phosphorylation. Rat kidney homogenate (75 μg) was incubated at 25°C for 30 min in the presence of 50 μM [γ-^{32}P]ATP; and catalytic subunit of protein kinase A (Calbiochem) in phosphorylation buffer containing 20 mM Tris–HCl (pH 7.4); 100 mM NaCl; 5 mM $MgCl_2$; 5 mM NaH_2PO_4; 1.5 mM $CaCl_2$; 0.2% (v/v), Triton X-100; 1 mM EDTA; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride; 5 μg/ml each of leupeptin, pepstatin, and antipain. At the end of incubation period the phosphorylation reaction was stopped by immunoprecipitation with AQP1 antibody as described below.

Immunoprecipitation of phosphorylated AQP1. Phosphorylated homogenate was incubated with 10 mg of preswollen Protein A-Sepharose beads and incubated for 1 h at 4°C. The Sepharose beads associated, nonspecifically adsorbed proteins were removed by centrifugation for 10 s at 15,000 rpm in microcentrifuge. The supernatant was then mixed with 2 μl of AQP1 antibody (Alomone Labs, Jerusalem, Israel) and the mixture was incubated for 12 h at 4°C. The samples were then transferred to Eppendorf tube containing 10 mg of preswollen Protein A-Sepharose beads and incubated for 1–2 h at 4°C. The beads were collected by centrifugation and washed once with lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA; pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride; 5 μg/ml each of leupeptin, pepstatin, and antipain); three times with 1 ml buffer containing 20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA (pH 8.0) and once with 1 ml of a buffer containing 50 mM Tris–HCl (pH 8.0). After the final wash, the beads were resuspended in 50 μl of SDS–PAGE sample buffer (50 mM Tris–HCl, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), vortexed and centrifuged. The recovered proteins were separated by 12% SDS–PAGE. Gels were dried and subjected to autoradiography.

RESULTS AND DISCUSSION

AQP1 Is Phosphorylated in Vitro by cAMP-Dependent Protein Kinase A

We performed the experiments in which *in vitro* phosphorylation of AQP1 was carried out as described for AQP4 (32). Briefly, rat kidney homogenate (75 μg) was incubated with catalytic subunit of PKA and [γ-^{32}P]ATP and the phosphorylated proteins were immunoprecipitated with AQP1 specific antibody purchased from Alomone Labs (Jerusalem, Israel). Immunoprecipitated proteins were separated by SDS–PAGE and analyzed by autoradiography to judge the phosphorylation of AQP1 protein. We observed a major band with an apparent molecular mass of 28 kDa and a minor band with an apparent molecular mass of 35–40 kDa (Fig. 1). The 28-kDa band corresponds to unglycosylated protein and 35- to 40-kDa band corresponds to glycosylated proteins. The intensity of phosphorylated bands in the presence of catalytic subunit of PKA (lane 2) was significantly higher than the bands in the absence catalytic subunit of PKA (lane 1) or in the presence of PKA inhibitor (Sigma Chemical Co.) (lane 3). Analysis of Fig. 1 by densitometry showed that the density of the 28-kDa band in the presence of PKA was 11 times higher than of that in the absence of PKA or in the presence of PKA inhibitor. These results strongly suggested that both glycosylated and unglycosylated AQP1 peptides were phosphorylated by cAMP-dependent PKA *in vitro.* AQP1 lack the typical cAMP-

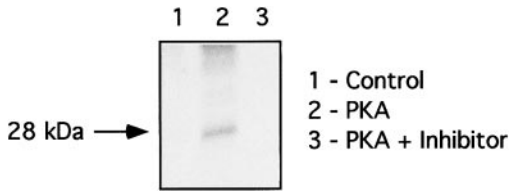

FIG. 1. *In vitro* phosphorylation of rat kidney AQP1 protein. Phosphorylation of AQP1 from rat kidney was identified as the incorporation of radioactivity into the protein in the presence or absence of PKA inhibitor of catalytic subunit of PKA. Autoradiogram was obtained after immunoprecipitation of phosphorylated AQP1 and separation of recovered proteins by SDS–PAGE electrophoresis. Similar results were obtained in two separate experiments using kidney homogenate from different rats.

dependent protein kinase consensus sequence Arg-Arg-X-Ser/Thr (where X is any amino acid) for phosphorylation. However, there are several proteins that are phosphorylated via cAMP-dependent protein kinase that exhibit only Arg-X-Ser sequence (33, 34). The Ser-238 of bovine AQP1 (28) utilized in the current experiments exhibits the Arg-X-Ser sequence that could be the potential phosphorylation site in this aquaporin. Further studies are necessary to identify the unique amino acid residue responsible for the phosphorylation of AQP1 and determine whether phosphorylation by cAMP-dependent PKA changes the activity or distribution pattern of AQP1 in native tissue. These studies will help us to delineate the molecular mechanisms involved in the regulation of AQP1 by cAMP and AVP.

Cyclic-AMP-Dependent Regulation of AQP1 Is via "Membrane Shuttle" Mechanism

Oocytes membranes were analyzed by immunoblot to study the effect of cAMP and AVP on the translocation of AQP1 from cytoplasm to membrane in oocytes. Oocytes were microinjected with 10 ng of *in vitro* transcribed AQP1 cRNA and membranes were isolated after 72 h as described under Methods. In some experiments, AQP1-expressing oocytes were treated for 30 min with 10 μM 8-bromo-cAMP (a membrane permeable analog of cAMP) or AVP prior to the membrane preparations. Our results obtained using the AQP1 antibody favors a cAMP-dependent membrane shuttle mechanism for AQP1 regulation. An unusual feature of AQP1 expression is that both glycosylated and nonglycosylated forms are present in native as well as in AQP1-expressing oocytes. Since there is a variation in glycosylation of AQP1 proteins in oocyte to oocyte the membranes from oocytes injected with AQP1 cRNAs were treated with *N*-glycosidase F to remove the N-linked carbohydrate moiety just prior to SDS–PAGE in order to simplify the quantitation of immunoreactive bands. We detected a band of an expected molecular mass of 28 kDa in AQP1 expressing oocytes as well as those treated with 8-bromo-cAMP or AVP for 30 min before membrane preparations when equal amounts of membrane protein (50 μg) were separated on SDS–PAGE (Fig. 2). Analysis of Fig. 2 by densitometry showed that the density of 28-kDa band corresponding to AQP1 in oocytes that were treated with 8-bromo-cAMP or AVP was 3–4 times higher than that of in untreated oocytes. These results further supports our earlier findings (29) of regulation of AQP1 by cAMP and AVP. It is difficult to conclude whether the increased AQP1 protein level in oocyte membranes is a direct response to AVP-induced intracellular signaling or to indirect effects of AVP. Although it has been well know that functions of many channel proteins are modulated through protein phosphorylation (35), our results indicate that the translocation of the channel protein may be regulated by phosphorylation. A recent report (36) demonstrates that AVP augmented the AQP1 expression in the NaCl-stressed mouse inner medullary collecting duct cells in which AQP1 was found to be translocated from cytosol to membrane in response to AVP under osmotic stress. Similarly, water channel activity of AQP2 in kidney is also regulated by AVP via cAMP-dependent "membrane shuttle" mechanism in which AVP increases the apical membrane water permeability by triggering exocytosis of intracellular vesicles containing AQP2 to the apical membrane (37, 38).

The regulation of AQP1 is significant clinically since the knockout mice studies with AQP1 suggest that it is involved in several fluid transporting epithelia such as proximal tubule and descending limb of Henle epithelia in kidney and microvascular endothelia in lung (17, 21). In eye, its presence in the ciliary body (26, 28, 39), and trabecular meshwork may contribute to aqueous humor production and elevated intraocular pressure as occurs in glaucoma. Further characterization of aquaporin function at the molecular level in aqueous inflow and outflow pathways should provide new insights into normal physiology and disease mechanisms, and may

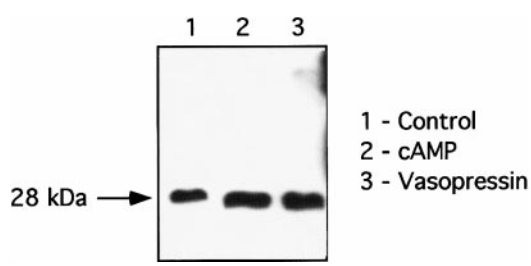

FIG. 2. Effect of c-AMP and AVPO on the changes in the amount of AQP1 in the AQP1-expressing oocyte membranes. Oocyte membranes were treated with *N*-glycosidase F to remove the carbohydrate moiety prior to SDS–PAGE separation. After SDS–PAGE separation of membranes, the proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-AQP1 antibody. A typical Western blot of AQP1 in the oocyte membranes is shown.

yield novel therapies to regulate fluid movement in glaucoma. At the least, our results provide mounting evidence that, in addition to AQP2, other aquaporins such as AQP1 are likely amenable to pharmacological regulation and furthermore such activity appears to be of physiologic relevance.

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