Regulation of Aquaporin-4 Water Channels by Phorbol Ester-dependent Protein Phosphorylation*

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Zhiqiang Han, Martin B. Wax, and Rajkumar V. Patil†
From the Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

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. However, because humans with defective AQP1 are phenotypically normal and because the ocular application of phorbol esters reduce intraocular pressure, we postulated that the water channel activity of AQP4 may be regulated by these agents. We now report that protein kinase C (PKC) activators, phorbol 12,13-dibutyrate, and phorbol 12-myristate 13-acetate strongly stimulate the phosphorylation of AQP4 and inhibit its activity in a dose-dependent manner. Phorbol 12,13-dibutyrate (10 μM) and phorbol 12-myristate 13-acetate (10 nM) reduced the rate of AQP4-expressing oocyte swelling by 87 and 92%, respectively. Further, phorbol 12,13-dibutyrate significantly increased the amount of phosphorylated AQP4. These results demonstrate that protein kinase C can regulate the activity of AQP4 through a mechanism involving protein phosphorylation. Moreover, they suggest important potential roles for AQP4 in several clinical disorders involving rapid water transport such as glaucoma, brain edema, and swelling of premature infant lungs.

Aquaporins are a rapidly growing family of water channel proteins found in animals, plant, and microorganisms (1, 2). At least eight different aquaporins have been identified and cloned from mammals, including AQP1 from erythrocytes (3, 4), AQP2, AQP3, and AQP6 from kidney (5–7), AQP4 from brain (8, 9), AQP5 from salivary gland, (10), AQP7 from testis (11), and AQP8 from testis and liver (12, 13). AQP1, AQP2, AQP3, and AQP7 have been shown to transport the nonionic small solutes such as urea and glycerol in addition to water (6, 11, 14), whereas AQP4, AQP5, AQP6, and AQP8 are highly selective to water permeation and exclude small solutes (8, 9, 12). With the exception of kidney-specific, vasopressin-regulated AQP2, the aquaporins are thought to be constitutively active (15–18). The regulation of other aquaporins is controversial (see “Discussion”) and is not well understood.

The ciliary body expresses only two aquaporins (AQP1 and AQP4). Because humans with mutation defects in AQP1 are phenotypically normal (19) and because the application of phorbol esters to the eye reduces intraocular pressure (20), we postulated that phorbol ester regulation of AQP4 water channel activity may account for the observed reduction of intraocular pressure using these agents. AQP4 is unique because it encodes a water-selective channel that is not inhibited by high concentrations of mercurial compounds such as HgCl₂ (9). Previous studies using immunocytochemistry, reverse transcription polymerase chain reaction, and Northern blotting with AQP4 confirmed its expression in kidney, brain, lung, and eye including retina and ciliary body (21–23). Using an oocyte swelling assay and protein phosphorylation studies, we demonstrate here that water channel activity of AQP4 is regulated by phorbol ester-dependent protein phosphorylation via protein kinase C (PKC) pathway. AQP4 regulation by PKC suggests an important potential role for this aquaporin in several clinical disorders involving rapid water transport such as glaucoma, brain edema following stroke, and uncontrollable swelling of premature infant lungs.

EXPERIMENTAL PROCEDURES

In Vitro cRNA Synthesis of AQP4—The plasmid containing rat AQP4 cDNA (8) was purchased from ATCC (Rockville, MD). The EcoRI fragment of the plasmid containing entire AQP4 open reading frame was blunt-ligated into the BglII site of the Xenopus expression construct pXbG (3). Confirmation of the recombinant plasmid was made by nucleotide sequencing. Sense and antisense capped RNA transcripts of AQP4 were synthesized in vitro with T3 RNA polymerase using two recombinant plasmids with the AQP4 cDNA cloned in sense and anti-sense direction.

Preparation of Oocytes and Measurement of Pf—Defolliculated stage V and VI oocytes from female Xenopus laevis (24) were injected with 20 nl of water or cRNAs (1 mg/ml). After incubation in 200 mosmol modified Barth’s buffer at 18 °C for 72 h, oocytes were transferred to 70 mosmol Barth’s buffer diluted with distilled water, and the time course of osmotic volume increase was monitored at 20 °C. Because the time course of cell swelling was principally linear during the initial 40 s, osmotic water permeability (Pf) of oocytes was calculated from this 40-s response as described previously (25). The effects of phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA), 4a-phorbol, 4α-phorbol 12,13-didecanoate (4α-PDD), (all from Calbiochem), and HgCl₂ were examined by incubating oocytes in Barth’s buffer containing appropriate concentrations of the reagent for 15 min prior to Pf measurements.

In Vitro Phosphorylation—Rat brain homogenate (75 μg) was incubated at 25 °C for 30 min in the presence of 50 μM [γ-32P]ATP; 0.045 μg of PKC (Calbiochem) and PKC activators in phosphorylation buffer containing 20 mM Tris-HCl (pH 7.4); 100 mM NaCl; 5 mM MgCl₂; 5 mM NaH₂PO₄; 1.5 mM CaCl₂; 0.2% (v/v) Triton X-100; 1 mM EDTA; 1 mM dithiothreitol; 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 AQP4 antibody as described below.

Immunoprecipitation of Phosphorylated AQP4—Phosphorylated ho-

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† To whom correspondence and reprint requests should be addressed: Dept. of Ophthalmology and Visual Sciences, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110. Tel.: 314-362-3770; Fax: 314-362-3638; E-mail: patil@am.seer.wustl.edu.

1 The abbreviations used are: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PAGE, polyacrylamide gel electrophoresis.
Effect of Phorbol Esters on AQP4 Water Channel Activity—An expression construct was prepared by inserting the AQP4 coding sequences between 5′- and 3′-untranslated sequences of the Xenopus ß-globin cDNA as described under “Experimental Procedures.” Defolliculated oocytes were microinjected with 20 ng of AQP4 cRNA 72 h prior to experiments. Oocytes were incubated with PDBu or 4α-phorbol as described under “Experimental Procedures.” After the treatments, osmotic swelling of the oocytes was monitored, and oocyte volume was calculated. Each point represents the mean ± S.E. of 8–12 oocytes. Similar results were obtained in three separate experiments with oocytes from different frogs.

AQP4 is a mercury-insensitive water channel (8, 9). Oocytes injected with water or antisense cRNA showed a very low swelling rate that was unaffected by either PDBu or PMA treatment.

The coefficients of osmotic water permeability (Pf) at 20 °C calculated from the rates of swelling were 32.4 ± 2 μm/s (mean ± S.E., n = 8) for untreated AQP4 injected oocytes and 4.3, 5.3, and 9.1 ± 1 μm/s (mean ± S.E., n = 12) for PDBu-treated oocytes at 10, 5, and 1 μM concentrations, respectively (Fig. 3), whereas the Pf values of oocytes incubated with 4α-phorbol were 51 ± 2 μm/s (mean ± S.E., n = 8), suggesting that the decrease in Pf of AQP4 due to PDBu was specific. In another experiment, similar results were obtained using PMA. The Pf values of oocytes incubated with PMA at 10, 5, and 1 μM, were 2.4, 8.2, and 13 ± 2 μm/s (mean ± S.E., n = 10), respectively, whereas the Pf values of untreated oocytes and 4α-PDD-treated oocytes were 29 and 24.9 ± 3 μm/s (mean ± S.E., n = 10), respectively. The Pf values of oocytes injected with water (data not shown) were 2.2 ± 1 μm/s (mean ± S.E., n = 8) suggesting that microinjection itself had no effect on the oocyte swelling. The phorbol ester-dependent decrease in water permeability suggests that AQP4 could participate in receptor-mediated regulation of water fluxes in a variety of tissues, such as kidney, heart, brain, lung, and eye, in which it is widely distributed (21, 22).

In Vitro Phosphorylation of AQP4—Activation of PKC by phorbol esters is known to stimulate the phosphorylation of several proteins, thereby modulating their function. Therefore, we tested whether phosphorylation of AQP4 could be achieved in vitro by PKC. For this purpose, we incubated equal aliquots of rat brain homogenate with or without PDBu in the presence of swelling in 70 mosmol buffer (Fig. 2) versus control oocytes. Oocytes incubated for 15 min in 200 mosmol Barth’s buffer containing 4α-PDD (inactive PMA) showed no effect on subsequent rate of swelling in 70 mosmol buffer. The effect of PMA was also dose-dependent; at 10 nM PMA reduced the rate of oocyte swelling by 92% in 70 mosmol buffer. Ethanol (0.1%) was used to make 1 mM stock solutions of PDBu, 4α-phorbol, PMA, and 4α-PDD and had no effect alone on swelling. Swelling of oocytes expressing AQP4 was not blocked by 1 mM HgCl2 (data not shown), because AQP4 is a mercury-insensitive water channel (8, 9). Oocytes injected with water or antisense cRNA showed a very low swelling rate that was unaffected by either PDBu or PMA treatment.

RESULTS AND DISCUSSION

Effect of Phorbol Esters on AQP4 Water Channel Activity

Fig. 1. Effect of PDBu on the osmotic water permeability of oocytes expressing AQP4 RNA. Oocytes were injected with 20 ng of AQP4 cRNA 72 h prior to experiments. Oocytes were incubated with PDBu or 4α-phorbol as described under “Experimental Procedures.” After the treatments, osmotic swelling of the oocytes was monitored, and oocyte volume was calculated. Each point represents the mean ± S.E. of 8–12 oocytes. Similar results were obtained in three separate experiments with oocytes from different frogs.

Significantly lower subsequent rate of swelling in 70 mosmol buffer (Fig. 2) versus control oocytes. Oocytes incubated for 15 min in 200 mosmol Barth’s buffer containing 4α-PDD (inactive PMA) showed no effect on subsequent rate of swelling in 70 mosmol buffer. The effect of PMA was also dose-dependent; at 10 nM PMA reduced the rate of oocyte swelling by 92% in 70 mosmol buffer. Ethanol (0.1%) was used to make 1 mM stock solutions of PDBu, 4α-phorbol, PMA, and 4α-PDD and had no effect alone on swelling. Swelling of oocytes expressing AQP4 was not blocked by 1 mM HgCl2 (data not shown), because AQP4 is a mercury-insensitive water channel (8, 9). Oocytes injected with water or antisense cRNA showed a very low swelling rate that was unaffected by either PDBu or PMA treatment.

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of γ-^32^P and PKC. After separation of immunoprecipitated proteins using AQP4-specific antibody, we observed two bands with an apparent molecular masses of 31 and 40–45 kDa (Fig. 4). The 31-kDa band corresponds to unglycosylated protein, and the 40–45-kDa band corresponds to glycosylated proteins. The intensity of phosphorylated bands in the presence of PDBu was significantly higher than the bands in the absence of PDBu or in the presence of PKC inhibitor or 4a-phorbol. Analysis of Fig. 4 by densitometry showed that the density of the 31-kDa band in the presence of PDBu was 12–12.5 times higher than that in the absence of PDBu or in the presence of PKC inhibitor or 4a-phorbol. These results strongly suggested that both glycosylated and unglycosylated AQP4 peptides were phosphorylated by PKC in the presence of PDBu.

Decreased oocyte Pf in AQP4 expressing oocytes by PDBu and PMA but not by inactive phorbol esters and increased ^32^P incorporation into AQP4 in in vitro phosphorylation in the presence of PDBu but not in the presence of inactive phorbol or PKC inhibitor suggest that the phorbol ester-dependent phosphorylation of AQP4 is involved in the regulation of its water channel activity. Further studies are necessary to identify the unique amino acid residue responsible for the phosphorylation of AQP4 and determine whether phosphorylation by phorbol ester-dependent PKC changes the activity or distribution pattern of AQP4 in native tissue. Thr-107, Ser-111, and Ser-180 (8) may be the potential phosphorylation sites for AQP4 because they are contained in the recognition motifs (S/T-X/R/K) or RXXS^* used by serine/threonine protein kinases such as PKC (26).

The regulation of aquaporins is the subject of major controversy. Recently, it was reported that forskolin stimulated the water channel activity of AQP1 (27). Although some investigators failed to reproduce these observations (28), we were able to duplicate these results in our laboratory (25, 29). Further, we have shown recently that AQP1 is regulated by arginine vasopressin and atrial natriuretic peptide in oocytes (25). Previous studies have shown that water channel activity of AQP2 is stimulated by cAMP-dependent protein phosphorylation (30). These results have also been controversial in oocyte (31) as well as in phosphorylation (32) studies using AQP2. Yet a recent report suggested that protein kinase activators such as forskolin and PMA had no effect on the water channel activity of AQP1, AQP2, AQP3, AQP4, or AQP5 expressing oocytes and concluded that phosphorylation is not involved in the regulation of these proteins (33). Some of these discrepancies may be due to variation in oocyte batches undertaken for the studies. For example, studies with activation of ionic currents in Xenopus oocytes by arginine vasopressin showed that not all donor frogs are responsive to this peptide, and the response was variable between oocytes from a single donor (34). Furthermore, these studies indicated that there may be a seasonal variation in expression of the receptors for these neuropeptides.

The regulation of AQP4 is likely significant clinically because it is a major water channel protein in brain and may therefore play an important role in the swelling that follows stroke. In lung, where there is a sharp increase in AQP4 expression just after birth (35) it may play an important role in the clearance of fluid from the newborn lungs. In eye, its presence in the ciliary body (9, 23, 36) may contribute to aqueous humor production and elevated intraocular pressure as occurs in glaucoma. Furthermore, its presence in retinal Muller cells may contribute to visual function by its involvement in the light-dependent hydration of space-surrounding photoreceptors (see Ref. 37). In addition, recent evidence demonstrates that AQP4 knockout mice have impaired ability to concentrate urine, suggesting a functional role for this aquaporin in the kidney (38). At the least, our results provide mounting evidence that in addition to AQP2, other aquaporins such as AQP4 are likely amenable to pharmacological regulation and furthermore such activity appears to be of physiologic relevance.

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FIG. 3. Changes in Pf of oocytes expressing AQP4 RNA after PDBu and PMA treatments. Oocytes were injected with 20 ng of AQP4 cRNA 72 h prior to experiments. Oocytes were incubated with PDBu or PMA as described under “Experimental Procedures.” After the treatments, osmotic swelling of the oocytes was monitored, and Pf was calculated. Pf is shown as the mean ± S.E. of 8–12 oocytes. Similar results were obtained in three separate experiments with different batches of oocytes.

FIG. 4. Effect of PDBu on the phosphorylation of rat brain AQP4 protein. Phosphorylation of AQP4 from rat brain was identified as the incorporation of radioactivity into the protein by PKC in the presence of 5 μM of PDBu (with or without PKC inhibitor) or 4a-phorbol for 30 min. Autoradiogram was obtained after immunoprecipitation of phosphorylated AQP4 and separation of recovered proteins by SDS-PAGE. Similar results were obtained in two separate experiments using brain homogenate from different rats.
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