Water permeability of aquaporin-4 is decreased by protein kinase C and dopamine

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Zelenina, Marina, Sergey Zelenin, Alexander A. Bondar, Hjalmar Brismar, and Anita Aperia. Water permeability of aquaporin-4 is decreased by protein kinase C (PKC) and dopamine. Am J Physiol Renal Physiol 283: F309–F318, 2002. First published February 19, 2002; 10.1152/ajprenal.00260.2001.—Aquaporin-4 (AQP4) plays an important role in the basolateral movement of water in the collecting duct. Here we show that this water channel can be dynamically regulated. Water permeability (Pf) was measured in individual LLC-PK1 cells that were transiently transfected with AQP4. To identify which cells were transfected, AQP4 was tagged at the NH2 terminus with green fluorescent protein. Transfected cells showed a strong fluorescent signal in basolateral membrane and a low-to-negligible signal in the cytosol and apical membrane. Activation of protein kinase C (PKC) with phorbol 12,13-dibutyrate (PDBu) significantly decreased Pf of cells expressing AQP4 but had no effect on neighboring untransfected cells. No redistribution of AQP4 in response to PDBu was detected. Dopamine also decreased the Pf in transfected cells. The effect was abolished by the PKC inhibitor Ro 31–8220. Reduction of AQP4 water permeability by PDBu and dopamine was abolished by point mutation of Ser180, a consensus site for PKC phosphorylation. We conclude that PKC and dopamine decrease AQP4 water permeability via phosphorylation at Ser180 and that the effect is likely mediated by gating of the channel.

METHODS

DNA constructs. Constructs encoding the short form of AQP4, AQP4-M23 (64), were used. cDNA encoding the whole AQP4-M23 was amplified by RT-PCR using total mouse brain RNA as a template. The cDNA fragments were subcloned in the frames of pEGFP-N1 and pEGFP-C2 plasmids (Clontech, Palo Alto, CA). The termination codon between AQP4-M23 and GFP in pEGFP-N1 was eliminated by using a U.S.E. Mutagenesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Inserts in the constructs expressing COOH- or NH2-terminal GFP-tagged AQP4 fusion protein were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, U.K.). Analysis of AQP4 protein structure to predict PKC phosphorylation sites and to estimate their phosphorylation potential was performed using NetPhos 2.0 software (2). The point mutation of Ser180 to alanine (S180A) was performed using computer software (2).

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achieved 80% confluence on the fourth day of culture, when the cells did not express AQP4. The cells were transfected on the second day of culture as described above. Experiments were performed at the fourth day of culture, when the cells achieved 80–90% confluence.

In some experiments, LLC-PK1 cells were grown on permeable supports (0.2-μm Anopore membrane; 10-mm tissue culture inserts; Nunc, Roskilde, Denmark). Transfection of the cells was performed on the second day of culture as described above. Localization studies were performed on the fourth day of culture, when the cells reached confluence.

In one protocol, rat astrocytes (CTX TNA2, European Collection of Cell Cultures, Center for Applied Microbiology & Research; subpassages 3–11) were used. The astrocytes were grown on coverslips in DMEM (Sigma-Aldrich Sweden) containing 0.5 U/ml penicillin and 50 μg/ml streptomycin supplemented with 10% FBS, 0.11 mg/ml sodium pyruvate, and 2 mM l-glutamine. According to our PCR studies, the astrocytes did not express AQP4. The cells were transfected on the second day of culture as described above. Experiments were performed on the fourth day of culture, when the cells achieved 80–90% confluence.

Measurement of water permeability. The water permeability was measured using a new approach, which combines a method that we have previously developed (65) with the use of proteins carrying fluorescent tags. In the beginning of every water permeability measurement, an image showing the distribution of GFP-tagged proteins was recorded (Fig. 1A). Then, with the specimen remaining on the stage of the microscope, the cells were loaded with calcein (Fig. 1B) and the water permeability measurement was performed (see details below). During the off-line analysis of the series of the calcein images, the GFP and calcein images were superimposed (Fig. 1C), which allowed us to identify the transfected cells and to calculate the water permeability separately for cells that did and did not express AQP4.

The coverslips with the cells were mounted in a closed perfusion chamber (Focht live cell chamber system, Butler, PA) on the stage of a Zeiss 410 invert laser scanning microscope. The cells were scanned using a ×63/1.4 oil-immersion objective. Fluorescence was excited by a 488-nm argon laser line, and emitted fluorescence was detected using a bandpass filter at 515–525 nm. The intensity of the excitation light necessary for GFP imaging was at least 100-fold higher than that for imaging of calcein fluorescence. The GFP fluorescence, therefore, did not have any significant influence on water permeability measurements.

The cells were loaded with calcein by a 5-min exposure to 20 μM calcein-acetoxymethyl ester (calcein-AM; Molecular Probes Europe, Leiden, The Netherlands) in 300 mosM PBS (in mM: 137 NaCl, 0.9 CaCl₂, 0.49 MgCl₂, 2.7 KCl, 1.5 KH₂PO₄, 8.1 Na₂HPO₄, pH 7.35). The loading solution was changed to 300 mosM PBS containing drugs or vehicle, and the cells were incubated for 3 min with 10⁻⁶ M phorbol 12,13-dibutyrate (PDBu; Sigma-Aldrich Sweden) or 10⁻⁵ M dopamine (Intropin; DuPont Pharmaceuticals; Meda, Täby, Sweden). In experiments with PKC inhibition, the cells were incubated with 10⁻⁷ M Ro 31–8220 (Calbiochem, La Jolla, CA) or vehicle for 10 min before incubation with dopamine. The loading of the cells with calcein and incubation with the drugs was performed at 30°C. The subsequent water permeability measurements were carried out at 10°C to decrease the diffusional water movement through the lipid bilayer of the plasma membrane. To measure water permeability, the cells were perfused with cold 300 mosM PBS for ~1 min; then, the perfusate was switched to 150 mosM PBS at the same temperature (in mM: 62 NaCl, 0.9 CaCl₂, 0.49 MgCl₂, 2.7 KCl, 1.5 KH₂PO₄, 8.1 Na₂HPO₄, pH 7.35). It has to be noted that at subconfluence LLC-PK1 cells do not have a tight contact with a coverslip and each other (Fig. 2), which allows access of the perfusion solution to the basolateral surface of the cells. Confocal images were recorded every 2 s before and after the solution switch. The series of images were analyzed off-line by measuring the time course of the calcein fluorescence in cytoplasmic regions of individual cells. The part of...
the obtained curves recorded immediately after the solution switch was used for water permeability calculation. During this period (~10 s), cell swelling is believed to be proportional to the permeability of the cellular membranes to water and is not influenced by mechanisms aimed at regulatory volume decrease.

Water permeability was calculated using the following equation (11, 65)

\[ P_t = \tau (1 - b/V_o) \left[ \gamma (A/V)_0 V_w \Delta \phi_0 \right]^{-1} \]

where \( P_t \) is water permeability. The time constant \( \tau \) is calculated for every cell from the curve, showing changes in fluorescence intensity inside the cell during osmotic swelling. It was calculated as the time constant of the fitting of the initial part of the curve by a single exponential function, and \((1 - b/V_o)\) represents the osmotically active portion of the cell volume. This portion was calculated using direct volume measurements performed on the same cells before and after osmotic swelling (65). The measurements were done using the Imaris image-processing toolkit (Bitplane, Zurich, Switzerland) on stacks of images recorded through the calcein-loaded cells at 300 mosM and after complete swelling in 150 mosM PBS. For the LLC-PK1 cells, the osmotically active portion of the cell volume was found to be 0.77 ± 0.06 (n = 10), and for astrocytes it was 0.32 ± 0.05 (n = 7). The constant \( \gamma \) was calculated as the slope of the relative fluorescence vs. the relative osmolarity calibration curve. For the LLC-PK1 cells, it was found to be 0.78, and for astrocytes it was 0.42. \((A/V)_0\) is the initial cell surface-to-volume ratio. It was calculated using Imaris on stacks of images recorded through the calcein-loaded cells incubated in 300 mosM PBS. For the LLC-PK1 cells, the ratio was found to be 7,827 ± 286 cm⁻³ (n = 27), and for astrocytes it was 6,767 ± 315 cm⁻³ (n = 7). The osmotically active portion of the cell volume, the constant \( \gamma \), and the initial cell surface-to-volume ratio were determined at 10°C. \( V_w \) is the partial molar volume of water (18 cm³/mol), and \( \Delta \phi_0 \) is the initial osmotic gradient (outside – inside), which in our experiments was 1.5 × 10⁻⁴ mol/cm³.

The basal level of water permeability of LLC-PK1 cells could vary depending on the lot of CLONfectin used for the transfection. It also varied with the cell culture subpassage. Therefore, we only compared the water permeability of vehicle- and drug-treated cells that were at similar subpassage and were transfected with the same CLONfectin preparation.

Analysis of the subcellular distribution of GFP-tagged AQP4 proteins. Coverslips with transfected cells were mounted in the same chamber as was used for water permeability measurements (see above) in 300 mosM PBS. A stack of images with a vertical displacement of 0.4 μm were recorded. Then, the solution was changed to 300 mosM PBS containing 10⁻⁶ M PDBu, the cells were incubated for 3 min, and a new stack of images was recorded. The ratio of GFP signal in the plasma membrane to that in adjacent cytosol was measured using Scion Image software (Scion, Frederick, MD). The subcellular localization of AQP4 was compared in the same cells before and after treatment with PDBu. Images from the same cell height were taken for the comparison. No swelling or shrinkage of the cells was observed in response to PDBu.

Filters with confluent LLC-PK1 cells were placed on a coverslip in a drop of 300 mosM PBS. A stack of images were recorded, as described above, before and after addition of an equal volume of 300 mosM PBS containing 2 × 10⁻⁶ M PDBu.

Data presentation and analysis. Data are presented as means ± SE. Statistical analyses were made by using Student’s t-test. A difference of \( P < 0.05 \) was considered statistically significant.

RESULTS

Subcellular distribution and water permeability of AQP4 tagged with GFP at the NH₂ or COOH terminus. The subcellular distribution of AQP4 tagged with GFP was studied using confocal microscopy. NH₂-terminal GFP-tagged AQP4 expressed in the LLC-PK1 cell line was targeted specifically to the plasma membrane of the cells (Fig. 3A) with very low signal present in cytoplasm. The XZ projection image (Fig. 3B) shows that the protein is localized exclusively in basal and lateral membranes of the cells. COOH-terminal GFP-tagged AQP4 was also targeted to the basolateral membranes of LLC-PK1 cells (Fig. 3, E and F) but was to a fairly large extent present in the cytoplasm of the cells.

LLC-PK1 cells expressing either NH₂- or COOH-terminal GFP-tagged AQP4 had significantly higher water permeability than untransfected LLC-PK1 cells from the same coverslips (Fig. 3, C, D, G, and H).

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To further test the functional properties of GFP-tagged AQP4, we used astrocytes, a cell type that expresses AQP4 in vivo and has a lower intrinsic water permeability than LLC-PK1 cells. The astrocyte cell line that we used did not express AQP4 according to our PCR studies (data not shown). The NH2-terminal GFP-tagged AQP4 was evenly distributed in the plasma membrane of the transfected astrocytes, with virtually no signal from cytoplasm (Fig. 3, I and J). In these cells, water permeability was increased more than fivefold compared with surrounding untransfected astrocytes (Fig. 3, K and L).

Effect of PDBu on water permeability of LLC-PK1 cells transfected with AQP4 tagged with GFP at the NH2 terminus. Further experiments were performed using NH2-terminal GFP-tagged AQP4, because its subcellular distribution resembled that in kidney collecting duct cells. The water permeability of LLC-PK1 cells transfected with NH2-terminal GFP-tagged AQP4 was significantly decreased in response to the PKC activator PDBu. No effect of PKC activation was observed in untransfected cells from the same coverslips (Fig. 4A).

To examine whether the decrease in water permeability caused by PKC activation may have been due to an internalization of the basolaterally located AQP4, the distribution of GFP-tagged AQP4 was examined in the same cells before and after treatment with PDBu.
We did not observe any change in the subcellular distribution of GFP-tagged AQP4 in response to PDBu in cells grown either on coverslips (Fig. 4, B and C) or on permeable supports (Fig. 4, D–G). The ratio of fluorescent signal in the plasma membrane to that in adjacent cytosol (M/C ratio) was calculated in the same region of the cell before and after treatment with PDBu. There were no detectable changes in the M/C ratio after PDBu treatment in cells grown on either coverslips or permeable supports (data not shown).
Water permeability of GFP-tagged AQP4 mutated at Ser\textsuperscript{180} (GFP-AQP4 S180A). AQP4 amino acid sequence analysis using NetPhos 2.0 software (2) has shown that the water channel has a phosphorylation site for PKC at Ser\textsuperscript{180}. High phosphorylation potential of this site raises a possibility that phosphorylation of Ser\textsuperscript{180} can mediate effects of PKC activation on the water permeability of AQP4. To test this hypothesis, the Ser\textsuperscript{180} residue was mutated to alanine. To confirm the mutation and absence of other modifications, the whole insert of GFP-AQP4 S180A construct was sequenced. The subcellular distribution of GFP-AQP4 S180A protein was similar to that of wild-type NH\textsubscript{2}-terminal GFP-tagged AQP4 (Fig. 5, A and B). Most of the signal was present in the basolateral plasma membrane, with weak background fluorescence inside the cells. The water permeability of the cells expressing GFP-AQP4 S180A was similar to that of the cells that expressed wild-type GFP-tagged AQP4 (Fig. 5C), indicating that the mutation per se did not change the water channel properties of AQP4. The basal level of water permeability in this and following experiments was somewhat lower than in the previous series. This decrease is related to the change of the lot of CLONfectin used for transfection (see Methods).

The negative effect of PDBu on water permeability of cells that expressed wild-type GFP-AQP4 (Fig. 4A) was abolished in cells that expressed the mutated water channel GFP-AQP4 S180A (Fig. 5D). This suggests that the phosphorylation site at Ser\textsuperscript{180} is involved in the regulation of AQP4 by PKC activation.

**Effect of dopamine on the water permeability of wild-type and Ser\textsuperscript{180}-mutated NH\textsubscript{2}-terminal GFP-tagged AQP4.** In cells that expressed GFP-AQP4, the water permeability was significantly decreased by dopamine (Fig. 6A). In pilot studies, we have found that in the concentration used \((10^{-5}\text{ M})\), dopamine specifically activates the dopamine receptors in LLC-PK\textsubscript{1} cells and has no effect on other catecholamine receptors. There was no effect of dopamine on the water permeability of untransfected cells. Dopamine treatment had no effect on localization of GFP-AQP4. There were no detectable changes in the M/C ratio after dopamine treatment in cells grown on coverslips (data not shown).

The decrease in the water permeability of wild-type GFP-AQP4 by dopamine was abolished by preincubation with the PKC inhibitor Ro 31–8220. Preincubation with the PKC inhibitor itself increased the water permeability in transfected cells, indicating that AQP4 might be constitutively phosphorylated by PKC to a certain level in untreated LLC-PK\textsubscript{1} cells (data not shown).

Dopamine did not have any effect on the water permeability of the cells that expressed mutated GFP-AQP4 S180A protein (Fig. 6B).

**DISCUSSION**

*Evaluation of the method used for water permeability measurements.* Employment of confocal microscopy for the water permeability measurements (65) allows the study of the dynamic regulation of the water channels in differentiated mammalian cells. Because water permeability can be measured in individual cells, transient transfection of the cells with GFP-tagged water
channels can be used. Besides saving time, this allows comparison of the effects of experimental maneuvers in cells that express the water channel with those in neighboring untransfected cells.

Previous immunohistochemical studies have shown that in rats, AQP4 is localized on the basolateral membrane of principal cells in kidney collecting ducts (13, 14, 55), ependymal cells in the brain (13, 14, 47, 60), and airway epithelial cells in the lung (13, 31, 46). AQP4 distribution in astrocytes also has a highly polarized pattern (44, 47). In our experiments, AQP4 tagged with GFP at the NH2-terminal was targeted specifically to the plasma membrane of LLC-PK1 cells. COOH-terminal GFP-tagged AQP4 was also targeted to the plasma membranes but to a fairly large extent was present in the cytoplasm of the cells. This indicates that COOH-terminal GFP tagging can affect signals for plasma membrane targeting at the COOH terminus of the AQP4 molecule.

The basolateral targeting of AQP4 is not a hindrance for the water permeability studies in the LLC-PK1 cell line. According to our observations, the subconfluent LLC-PK1 cells do not have tight contacts with each other and with the coverslip (Fig. 2). Wide intercellular spaces allow the perfusion solution to reach the basolateral surface of the cells. The transmembrane osmotic water transport is therefore significantly increased when the cells express AQP4 at the basolateral membrane.

In different experiments, the increase in water permeability in transfected cells compared with untransfected ranged from 1.5- to 2-fold, which is comparable to the raise of water permeability in LLC-PK1 cells after transfection with AQP1 or AQP2 (11, 29, 62). This relatively modest increase may be attributable to the high intrinsic water permeability of LLC-PK1 cells. In astrocytes, where the basal water permeability is substantially lower than in LLC-PK1 cells, the expression of AQP4 increases water permeability more than five-fold. We have observed in ongoing studies that application of first messengers, which activate signaling pathways other than the PLC-PKC pathway, can result in an up to fourfold difference in water permeability of transfected and untransfected LLC-PK1 cells. Taken together, these data indicate that the influence of unstirred layers is minor in our experimental setup.

It is the experience of our laboratory that LLC-PK1 cells transiently transfected with integral membrane proteins have a tendency to load with fluorophores (including calcein) to a somewhat lesser degree than neighboring untransfected cells. However, this does not compromise the water permeability measurements, because the change in calcein fluorescence after hyposmotic shock is calculated as relative to the initial fluorescence, and the obtained value does not by definition depend on the initial fluorescence intensity. Original signal-to-noise ratio, which is proportional to the initial fluorescence intensity, is lower in transfected than in untransfected cells (7.9 ± 0.4 and 10.7 ± 0.6, respectively; P < 0.001; n = 74 and 80 cells from 4 coverslips from independent experiments). However, in both groups of cells, the signal-to-noise ratio was high, and this difference did not lead to a higher variability of the data in transfected cells compared with untransfected cells.

Loading of the cells with calcein is achieved by incubation with calcein-AM, which, being electrically neutral, freely diffuses into the cells. Once inside the cell, this nonfluorescent substrate is converted by intracellular esterases into a polar membrane-impermeant fluorescent product that is retained by cells. Because neither transfected nor untransfected cells lose the fluorescent signal during the control period before osmotic shock or after cell swelling, we can exclude that the fluorescent esterase product is leaking out of the transfected cells more intensively than out of the untransfected cells.

Dynamic regulation of AQP4 in kidney epithelial cells. We show here that the activity of AQP4 can be dynamically regulated in a kidney epithelial cell line. Activation of PKC and dopamine decreased the water permeability of AQP4 but had no obvious effect on trafficking of the water channel. Mutation studies indicated that the downregulation of AQP4 water perme-
ability is mediated by phosphorylation of the protein at Ser \(^{180}\).

Reversible phosphorylation is perhaps the most common way by which the function of proteins can be physiologically altered (7). The activity of many ion channels and pumps has been shown to be regulated in this way (4, 53). Phosphorylation may alter the capacity of an integral membrane transporter either by an allosteric change that directly affects its permeability and/or by retrieval/recruitment of the protein from/to the plasma membrane. AQP2 is so far the only water channel in which functional regulation via phosphorylation reactions has been firmly established (6, 17, 26, 28, 36, 48). Phosphorylation of AQP2 influences trafficking but is not believed to have any gating effect (37). The results of the present study suggest that, in contrast to AQP2, AQP4 may be regulated via gating.

AQP4 has been shown to be phosphorylated by PKC in vitro (21), but the phosphorylation site was not identified. We analyzed the sequence of AQP4 and found that there is only one PKC site, Ser \(^{180}\) with considerable phosphorylation potential. When this residue was mutated into alanine, the PKC-mediated decrease in the water permeability of AQP4 was abolished. This suggests that the effect of protein kinase C on AQP4 water permeability is not due to phosphorylation of an intermediary protein but rather to direct phosphorylation of AQP4, resulting in an allosteric change of the molecule that leads to decreased water permeability.

AQP4 is expressed in principal cells of the collecting duct in the kidney (13, 14, 55). Deletion of AQP4 in mice leads to a defect in maximum urinary concentrating ability in response to water deprivation (5, 38). The water permeability of the basolateral membrane of collecting ducts is considerably higher than that of the apical plasma membrane (12). It has therefore been assumed that the apical membrane is the only rate-limiting barrier for water reabsorption in the collecting duct. However, studies of the AQP4 knockout mouse have indicated that the water channels in the basolateral membrane play a role in the regulation of the water reabsorption in the collecting duct (5). The observations that both in vivo and in vitro exposure of collecting ducts to AVP will cause a significant swelling of the collecting duct cells have also provided evidence for a barrier role of the basolateral membrane (12, 19, 32, 61). The present study shows that PKC activation can directly decrease permeability of AQP4 expressed in the basolateral membranes of kidney epithelial cells. This downregulation could represent a mechanism for repression of antidiuretic effects of AVP in collecting ducts by phorbol esters (1, 20), as well as by cholinergic and nucleotide receptor agonists (20, 33, 49).

There is strong evidence that dopamine can also counteract the effect of AVP in collecting ducts (35, 42, 53). Dopamine actions are mediated via two types of receptors, D\(_1\) and D\(_2\), both of which have been shown to be able to signal via the phospholipase C and PKC pathway (reviewed in Ref. 41). Data from the present study indicate that dopamine will, by PKC-dependent downregulation of AQP4 water permeability, decrease the water permeability of the basolateral membrane of collecting duct epithelial cells. This should lead to a decrease in the efficacy of AVP-stimulated water reabsorption in this segment of nephron.

In basolateral membranes of kidney collecting duct, AQP4 is coexpressed with another member of the aquaporin family, aquaporin-3 (AQP3) (10, 24). The relative contribution of these two water channels to the basolateral water permeability of the collecting duct remains to be determined. Recent studies indicate that the water permeability of AQP3 can be downregulated by acidic pH (66). Coexistence of these two water channels with presumably different dynamic regulation in the same membrane would give the principal cells an opportunity to regulate the water reabsorption in various conditions.

Many cases of therapy-resistant water retention can be attributable to enhanced water permeability of the apical membrane of the collecting duct cells (34, 51). Further studies of factors that can downregulate AQP4 water permeability in the kidney might lead to new therapeutic strategies for the treatment of this condition.

The results from this study may also have important implications for the physiological role of AQP4 in several other tissues. AQP4 is expressed in astrocytes and meningeal and ependymal cells in the brain (13, 14, 44, 47), where it has been proposed to play a role in the regulation of water permeability at the blood-brain barrier, in the regulation of brain interstitial fluid composition, and in osmosensory processes (25, 30, 39, 43, 44, 54, 57–59). It is also expressed in the epithelial cells of the trachea and terminal bronchi (13, 46), where it has been suggested to participate in lung-water clearance (31, 56, 63). In skeletal muscle, AQP4 is expressed in the sarcolemma of fast-twitch fibers and is supposed to be involved in the pathogenesis of muscular dystrophy (15, 16). The results of the present study may imply that activation of the PKC signaling pathway may have a negative effect on lung water clearance and osmotic equilibration in exercising skeletal muscle and may modulate brain water transport.

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