Aquaporin-1 Channel Function Is Positively Regulated by Protein Kinase C*

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Aquaporin-1 (AQP1) channels contribute to osmotically induced water transport in several organs including the kidney and serosal membranes such as the peritoneum and the pleura. In addition, AQP1 channels have been shown to conduct cationic currents upon stimulation by cyclic nucleotides. To date, the short term regulation of AQP1 function by other major intracellular signaling pathways has not been studied. In the present study, we therefore investigated the regulation of AQP1 by protein kinase C. AQP1 wild type channels were expressed in Xenopus oocytes. Water permeability was assessed by hypotonic challenges. Activation of protein kinase C (PKC) by 1-oleoyl-2-acetyl-sn-glycerol (OAG) induced a marked increase of AQP1-dependent water permeability. This regulation was abolished in mutated AQP1 channels lacking both consensus PKC phosphorylation sites Thr157 and Thr239 (termed AQP1 ΔPKC). AQP1 cationic currents measured with double-electrode voltage clamp were markedly increased after pharmacological activation of PKC by either OAG or phorbol 12-myristate 13-acetate. Deletion of either Thr157 or Thr239 caused a marked attenuation of PKC-dependent current increases, and deletion of both phosphorylation sites in AQP1 ΔPKC channels abolished the effect. In vitro phosphorylation studies with synthetic peptides corresponding to amino acids 154–168 and 236–250 revealed that both Thr157 and Thr239 are phosphorylated by PKC. Upon stimulation by cyclic nucleotides, AQP1 wild type currents exhibited a strong activation. This regulation was not affected after deletion of PKC phosphorylation sites in AQP1 ΔPKC channels. In conclusion, this is the first study to show that PKC positively regulates both water permeability and ionic conductance of AQP1 channels. This new pathway of AQP1 regulation is independent of the previously described cyclic nucleotide pathway and may contribute to the PKC stimulation of AQP1-modulated processes such as endothelial permeability, angiogenesis, and urine concentration.

The aquaporins (AQPs)3 are a family of integral membrane proteins that are widely expressed in bacteria, plants, and animals (1). Their main physiological function is rapid transmembrane transport of water driven by osmotic gradients. In addition to water transport, some aquaporins also conduct ion currents. In humans, 11 aquaporins have been found so far (AQP0–AQP10). Of these, AQP1 is the predominant and least specialized subtype. It plays a major role in constitutive water transport through the membranes of several cell types including endothelial cells, red blood cells, and renal proximal tubule cells (1). It has been shown that AQP1 may also function as a cyclic nucleotide-gated cation channel that is activated mainly by cGMP and indirectly also by cAMP (2, 3). Recently, ion currents of native aquaporins were confirmed in choroid plexus epithelium and shown to modulate fluid transport of those cells (4).

Because of its significance for determining endothelial water permeability, AQP1 has been found to play a major physiological role in the peritoneal membrane (5). Subsequently, it has been shown to be the molecular correlate of the “ultrasmall pore” responsible for transcellular water permeability during peritoneal dialysis (6). Furthermore, recent reports have demonstrated a previously unexpected role of AQP1 in cell migration (7–9). Endothelial cells lacking AQP1 have impaired cell motility because of reduced formation of lamellipodia, resulting in impaired angiogenesis (7). Interestingly, AQP1 was also found to be essential for normal migration of renal proximal tubule cells and restitution of renal injury (8, 9).

It is well documented that aquaporin channels may be subject to intense short term regulation by cellular signal cascades, with the most recognized example being the cAMP-dependent protein kinase-dependent regulation of AQP-2 in the kidney collecting duct (1, 10). Interestingly, however, little is known to date about the short term regulation of AQP1, because this channel was originally considered to be constitutively open (1). The role of signaling pathways apart from cyclic nucleotides in the regulation of AQP1 has not been investigated to date.

The protein kinase C (PKC) system is a key component of intracellular signaling, and PKC activation is a central pathway downstream of Gq/11 coupled receptors such as adrenergic α1 receptors and muscarinic M1 receptors (11, 12). PKC has been shown to be involved in the regulation of structurally

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‡ The abbreviations used are: AQP, aquaporin; PKC, protein kinase C; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinepropanesulfonic acid; IBMX, isobutylmethylxanthine; CN, cyclic nucleotide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.
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diverse membrane proteins, particularly ion channels (13, 14). Recently, it has been demonstrated that PKC mediates the dopamine-dependent down-regulation of renal aquaporin-4 channels (15, 16). Notably, a major role of PKC in the regulation of endothelial permeability, angiogenesis, and water transport in the proximal renal tubule has been observed in physiological studies (17–24).

Here, we show that PKC positively regulates AQP1 channels expressed in Xenopus oocytes. PKC activation induces an increase of both water permeability and ion currents mediated by AQP1. On the molecular level, the effect depends on both consensus PKC phosphorylation sites Thr^{157} and Thr^{239}. To eliminate PKC-mediated phosphorylation at these positions, the two threonine residues were replaced with alanine. This resulted in the mutated channels T157A and T239A. Through repetitive mutagenesis, both point mutations were introduced into a single clone termed AQP1-ΔPKC. Point mutations were generated with the QuikChange protocol (Stratagene, La Jolla, CA). We used the primers CTCGTAATCCGACGCCCCG (forward) and CGCTTCCGCGCGTTATGCGACG (reverse) to introduce mutation T157A, and for mutation T239A, we used the primers CTCGTAATCCGACGCCCCG (forward) and CGCTTCCGCGCGTTATGCGACG (reverse). All of the cDNAs used in this study were verified by complete sequencing (Sequence Laboratories Göttingen GmbH).

Expression of AQP1 Channels in Xenopus Oocytes—The human AQP1 wild type clone was a kind gift from Peter Agre (Baltimore, MD). cRNA was prepared from the corresponding cDNA (AQP1 WT, AQP1-T157A, AQP1-T239A, and AQP1-ΔPKC) with T3 RNA polymerase after linearization with Smal using the mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX). AQP1 cRNA of wild type and mutant channels (always at the same concentration of 20 ng/μl) was injected into stage V and VI defolliculated oocytes using a Nanoject automatic injector (Drummond, Broomall, PA). The volume of injected cRNA solution was 46 nl/oocyte. The measurements were performed 2–3 days after expression.

Peptide Synthesis—Peptides corresponding to amino acid sequences 154–168 (P154–168, LATTDDRRRRDLGGSG, with a single conservative replacement of alanine by glycine at residue 168 to improve solubility) and to 236–250 (P236–250, SDDLTDVKKVWTSGEV, with a single conservative replacement of glutamine by glutamate at residue 249 to improve solubility) were obtained from Sigma-Genosys. Homologous peptides containing threonine-to-alanine mutations at residues 157 and 239, respectively, were obtained from the same source and referred to as P154–168 (T157A) and P236–250 (T239A), respectively.

In Vitro Phosphorylation Assays—10 μl (5 μg) of peptide and 25 ng of active PKC (Upstate Cell Signaling Solutions, Lake Placid, NY) were incubated at 30 °C for 15 min in the presence of ADBII, which was composed of 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithi-othreitol, 1 mM CaCl_2, 10 μl of lipid activator (1 mg/ml phosphatidyserine, 0.1 mg/ml diacylglycerol, 0.3% Triton X-100, 1 mM dithiothreitol), and 20 μM of [γ-32P]ATP (GE Healthcare, Munich, Germany) including 15 mM MgCl_2 and 200 μM unlabeled ATP in ADBII. The reactions were stopped by adding 4X SDS sample buffer, and the mixture was boiled for 5 min. The peptides were resolved by 20% SDS-polyacrylamide gel electrophoresis. The gels were dried and subjected to autoradiography using Hyperfilm MS film (GE Healthcare, Munich, Germany). The bands were quantified densitometrically with ImageJ on the basis of digitalized images.

Oocyte Membrane Isolation and Western Blotting Analysis—Noninjected and AQP1-cRNA-injected (200 ng/μl) Xenopus oocytes were used to isolate crude membrane fractions. 23 oocytes were incubated in 1000 μl of ice-cold hypotonic phosphate buffer (7.5 mM Na_2HPO_4, 1 mM EDTA, pH 7.5, plus a...
Protein kinase C activation increases water permeability of aquaporin-1 channels. In A, summary data of hypo-osmotic challenges are displayed. Relative cell volume measured digitally according to Anthony et al. (2) is shown as a function of time after transfer of the cell to the hypo-osmotic medium, i.e. to the observation period of 5 min. The volume of those cells is plotted as time course of cell swelling. Oocytes that did not express AQP1 completely lacked cell swelling (white squares; n = 7). In contrast, cells expressing AQP1 exhibited a time-dependent increase in cell volume as a consequence of water permeability (circles; n = 6). Activation of PKC by OAG in AQP1-expressing cells induced a markedly stronger cell swelling, indicating a marked increase in water permeability (squares; n = 8; p < 0.001). OAG was added to the hypo-osmotic medium; no preincubation was performed. Channels lacking both PKC phosphorylation sites (i.e. including mutations T157A and T239A) were termed AQP1-ΔPKC and measured under analogous conditions. Summary data of those experiments are shown in B. Under control conditions (i.e. without application of OAG), AQP1-ΔPKC channels induced an increase of relative cell volume in the hypo-osmotic medium by 9.8%. The addition of OAG to the medium did not induce any significant effect in those channels, resulting in a respective volume increase by 10.4% (n = 5 each, p >> 0.05).

RESULTS

Protein Kinase C Activation Increases Water Permeability of Aquaporin-1 Channels—Human AQP1 channels were expressed heterologously in Xenopus oocytes to allow measurement of water permeability and ion conductance. First, water permeability of the membrane of those cells was assessed by hypotonic swelling experiments according to Anthony et al. (2). The duration of these experiments had to be limited to 5 min (as in other studies (2)) to avoid cell destruction as a consequence of the volume increases. To activate PKC in a group of experiments, specific PKC activator OAG (a synthetic and more stable analogue of the physiological activator diacylglycerol) was added to the hypo-osmotic solution at a concentration of 10 μM. No preincubation of cells in OAG was performed. Summary data of the time course of volume change in those cells is plotted in Fig. 1A. Within 5 min, cell volume increased to 131.1 ± 2.6% (n = 8; p < 0.01 in comparison with control experiments without OAG). Without addition of OAG to the hypo-osmotic solution, cells expressing AQP1 exhibited a smaller volume increase to 115.3 ± 1.9% (n = 6; Fig. 1A; significantly different from the effect of OAG with p < 0.01). Cells that did not express AQP1 did not show any relevant volume change (100.3 ± 0.3%; n = 7; Fig. 1A; p < 0.001 in comparison with cells expressing AQP1).

Regulation of Aquaporin-1 Water Permeability by PKC Is Abolished in AQP1-ΔPKC Channels Lacking Phosphorylation Sites Thr157 and Thr239—Sequence analysis of AQP1 channel amino acid sequence revealed two consensus sites for PKC phosphorylation at Thr157 and Thr239, respectively. To elucidate the functional role of those sites, we modified channel subunits by site-directed mutagenesis. The respective threonin residue was replaced by alanine to abolish phosphorylation at the respective site. Channels lacking both PKC phosphorylation sites (i.e. including mutations T157A and T239A) were termed AQP1-ΔPKC and measured under conditions analogous to those described for wild type channels.

The results are summarized in Fig. 1B. After 5 min of exposure to the hypo-osmotic solution, the volume of oocytes expressing AQP1-ΔPKC channels increased to 109.8 ± 1.1% of the respective initial values (Fig. 1B, left column, n = 5). Then experiments were repeated, and OAG (10 μM) was added to the hypo-osmotic solution to activate PKC. In contrast to the effect observed in AQP1 wt channels, volume increase was not enhanced by PKC activation in AQP1-ΔPKC channels. After the observation period of 5 min, the volume of those cells increased to 110.4 ± 0.6% (Fig. 1B, right column, n = 5), i.e. values without significant difference to those observed without application of OAG (p >> 0.05). Therefore, we concluded that inactivation of both PKC phosphorylation sites abolishes the positive regulation of AQP1 water permeability by protein kinase C.

Protein Kinase C Activation Increases Aquaporin-1 Ion Currents—It has been demonstrated previously that AQP1 channels also conduct ions and that this conductance is activated by an increase in intracellular cyclic nucleotide levels, particularly cGMP and potentially also cAMP (2, 3). Hence, we examined whether the PKC system also regulates AQP1 ion currents.

A standardized voltage protocol was used to elicit AQP1 currents in Xenopus oocytes heterologously expressing these channels. From a holding potential of −30 mV that is close to the
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In A and B, representative recordings of AQP1 currents before and after application of PKC activator PMA (100 nM) for 30 min are shown. Corresponding I-V curves are superposed in C. Summary data of the observed effects are displayed as time course in D. In cells expressing AQP1, application of PMA induced a strong current increase that reached a plateau after ~20 min (black squares; n = 10). In contrast, no current increase was observed if no PMA was applied (black circles; n = 7). As shown in E, after the observation period of 30 min, PMA induced a relative current increase by 103% that was significantly different from the observations in the two control groups (p < 0.001 each). Protocol: holding potential −30 mV; test pulses from −120 mV to +60 mV in 10-mV increments (400 ms).

FIGURE 2. Protein kinase C activation by PMA increases ionic conductance of aquaporin-1 channels. In A and B, representative recordings of AQP1 currents before and after application of PKC activator PMA (100 nM) for 30 min are shown. Corresponding I-V curves are superposed in C. Summary data of the observed effects are displayed as time course in D. In cells expressing AQP1, application of PMA induced a strong current increase that reached a plateau after ~20 min (black squares; n = 10). In contrast, no current increase was observed if no PMA was applied (black circles; n = 7). As shown in E, after the observation period of 30 min, PMA induced a relative current increase by 103% that was significantly different from the observations in the two control groups (p < 0.001 each). Protocol: holding potential −30 mV; test pulses from −120 mV to +60 mV in 10-mV increments (400 ms).

FIGURE 3. Protein kinase C activation by OAG induces an increase of ionic conductance of aquaporin-1 channels that is suppressed by chelerythrine. Because PMA is not entirely specific for PKC, confirmatory experiments were performed with the use of the less potent, but more specific PKC activator OAG. Characteristic recordings of AQP1 currents before and after exposure to OAG (10 μM) for 30 min are shown in A and B. Corresponding I-V curves are plotted in C. The time course of effects is shown in D. OAG induced a marked current increase comparable with PMA. Co-application of PKC inhibitor chelerythrine (10 μM) suppressed this effect. Equally, no effect was observed if no OAG was added to the solution or if the cells did not express AQP1. As shown in E, the OAG-induced effect after 30 min was significantly different from the observations in the control groups (p < 0.001 each). Protocol: holding potential −30 mV; test pulses from −120 mV to +60 mV in 10-mV increments (400 ms).

reversal potential of AQP1 currents in low K+ solution, voltage steps to potentials from −110 mV to +60 mV (400 ms each) were applied. After recording a measurement under control conditions, phorbol ester PMA (100 nM) was perfused into the bath for 30 min to activate PKC. In contrast to the water permeability measurements, the electrophysiological experiments did not affect the integrity of the cells and could therefore be extended to an observation period of 30 min (instead of 5 min) that allowed a longer observation of the time courses. Measurements were recorded at intervals of 5 min until the end of the observation period. Outward current amplitudes during the step to +60 mV were determined and compared with quantified effects. Typical recordings under control conditions and after 30 min of exposure to PMA are shown in Fig. 2. Under control conditions, the cells merely exhibited small currents (Fig. 2A) with a reversal potential of approximately −30 mV and lack of inward or outward rectification (Fig. 2C). Application of PMA induced a marked increase of currents (Fig. 2B) without affecting reversal potential and rectification (Fig. 2C). During observation of the PMA effect, the currents increased exponentially and reached a plateau after ~20 min (Fig. 2D). Overall, a relative increase of current amplitudes by 102.5 ± 4.1% was observed (Fig. 2E; n = 10; p < 0.001 in comparison with control experiments). Control experiments were performed with oocytes that did not express AQP1. Those cells exhibited a merely small current increase (Fig. 2, D and E; n = 7; 14.2 ± 4.0%). For additional control experiments, oocytes expressing AQP1 were monitored for 30 min under identical conditions, but without the addition of PMA to the bath solution. Again, only a small current increase was observed in those cells (Fig. 2, D and E; n = 9, 17.1 ± 2.2%). PMA is a well established and highly potent activator of PKC, but it is less specific than OAG. There-
Phosphorylation Sites Thr\(^{157}\) and Thr\(^{239}\) Are Essential for Protein Kinase C Regulation of Aquaporin-1 Currents—Given that PKC regulation of AQP1 water permeability was found to depend on the availability of phosphorylation sites Thr\(^{157}\) and Thr\(^{239}\), we investigated the role of these residues for the PKC-induced current increase. Mutant channels AQP1-T157A, AQP1-T239A, and AQP1-ΔPKC (exhibiting both mutations in combination) were measured separately under conditions analogous to those described for wild type channels. OAG (10 \(\mu\)M) was used to activate PKC.

In Fig. 4 (A–C), typical recordings of AQP1-ΔPKC currents before and after exposure to OAG are displayed. Biophysical current properties were indistinguishable from the wild type. However, in contrast to wild type channels, AQP1-ΔPKC channels did not exhibit the strong activation induced by OAG.

Although there was a residual current increase by 41.2 ± 3.0% \((n = 7); \text{Fig. 4}; D \text{and} \ E\), the effect was dramatically weaker than in wild type channels \((p < 0.001)\). The channels in which only one of the two PKC phosphorylation sites had been inactivated (AQP1-T157A and AQP1-T239A) showed an attenuated activation by OAG (Fig. 4; D and E; \(p < 0.001\) in comparison with wild type). Relative current increases were 78.7 ± 3.6% in AQP1-T157A (Fig. 4; E; \(n = 9\)) and 78.6 ± 3.0% in AQP1-T239A (Fig. 4; E; \(n = 7\)), respectively. Thus, relative effects in those channels were comparable and ranged halfway between wild type and AQP1-ΔPKC (Fig. 4E), indicating that half of the PKC-dependent effect may be attributed to each of the two sites. Control experiments with AQP1-T157A, AQP1-T239A, and AQP1-ΔPKC channels without application of OAG showed a small current increase comparable with that of AQP1 wild type channels (Fig. 4; D and E).

Western blot analysis was performed with oocytes expressing AQP1 wild type and mutant channels. In membranes isolated from the same batch of oocytes, typical bands were found
Cyclic nucleotide induced activation of AQP1 conductance is independent of the protein kinase C pathway. It has been demonstrated previously that ionic conductance of AQP1 channels is positively regulated by cyclic nucleotides, mainly cGMP and potentially cAMP (2, 3). To test whether this pathway is still active in AQP1-ΔPKC channels lacking functional PKC phosphorylation sites, intracellular levels of cyclic nucleotides were increased by co-application of forskolin (100 μM) and IBMX (100 μM) to the cells. As shown in A-C, this induced a marked current increase after the observation period of 30 min. In line with previous reports, AQP1 wt currents also increased markedly upon exposure to forskolin and IBMX. The time course of effects is displayed in D. The summary data are shown in E. Forskolin and IBMX induced similar strong current increases in AQP1 wt channels (92%, n = 6) and in AQP1-ΔPKC channels (92%, n = 7), respectively (p > 0.05). The effect could be suppressed by co-application of guanylate cyclase inhibitor ODQ (50 μM) (14%, n = 6, p < 0.001). In control experiments performed without application of forskolin and IBMX, currents increased to 17% (n = 9) and 15% (n = 8), respectively (p < 0.001). Additional control experiments performed with cells that did not express AQP1 showed no effect, either (21%, n = 6). Thus, effects in AQP1 wt and in AQP1-ΔPKC channels were comparable, indicating that cyclic nucleotide-dependent activation of AQP1 is independent of the protein kinase C pathway. Protocol: holding potential −30 mV; test pulses from −120 mV to +60 mV in 10-mV increments (400 ms).

at the expected sizes with an identical pattern in wild type and mutant channels (Fig. 4D). Noninjected oocytes were used as controls (Fig. 4D, inset, left lane).

To examine phosphorylation of the two consensus sites that were found to be essential for the regulation of AQP1 by PKC in the functional measurements, we carried out in vitro phosphorylation. Two peptides were synthesized corresponding to the wild type amino acid sequences surrounding those sites: P154–168 and P236–250. In vitro, those peptides were phosphorylated by PKC (Fig. 4F). Additionally, two analogous peptides were synthesized that copied the corresponding region of the mutant channels AQP1-T157A and AQP1-T239A. The resulting peptides (referred to as P154–168(T157A) and P236–250(T239A)) were tested for phosphorylation under identical conditions. In contrast to the wild type sequences, PKC phosphorylation was absent in the mutant peptides (Fig. 4F).

Cyclic Nucleotide-induced Activation of AQP1 Currents Is Independent of the Protein Kinase C Pathway—It has been shown previously that both AQP1 ion conductance and water permeability may be positively regulated by cyclic nucleotides (2, 3). Having demonstrated that protein kinase C also regulates AQP1 positively, we were interested in examining whether there is an interaction between those two signaling pathways.

Therefore, AQP1-ΔPKC channels lacking functional PKC phosphorylation sites were expressed in Xenopus oocytes, and adenylate cyclase activator forskolin (100 μM) and phosphodiesterase inhibitor IBMX (100 μM) were co-applied to increase intracellular cyclic nucleotide levels. Electrophysiological experiments were performed with the same design as used for examining the PKC dependent regulation, i.e. with the same voltage protocol and the same observation time (30 min). The results are shown in Fig. 5. In AQP1 wild type channels, application of forskolin and IBMX induced a strong increase of currents by 96.8 ± 8.1% (n = 6; p < 0.001 compared with controls) after 30 min (Fig. 5, D and E). AQP1-ΔPKC channels exhibited a virtually identical response with a current increase by 92.1 ± 5.8% (n = 7; p < 0.001 compared with controls; Fig. 5, D and E). Co-application of guanylate cyclase inhibitor ODQ (50 μM) completely suppressed the effect, resulting in a small current increase by 13.9 ± 1.4% (n = 6; Fig. 5, D and E) that argues for a predominant role of cGMP in line with results from other groups (2, 4).

Control experiments were performed with cells that did not express AQP1 channels or with superfusion with the bath solution without forskolin and IBMX, respectively. In those experiments only small current increases within the limits of the nonspecific current run-up were observed (Fig. 5, D and E). Thus, we were able to reproduce the previously described positive regulation of AQP1 conductance by cyclic nucleotides in wild type channels with a predominant role of cGMP. Interestingly, mutated AQP1 channels lacking functional PKC phosphorylation sites exhibited a virtually identical response to this regulation, indicating that both pathways probably act independently on AQP1 channels.

DISCUSSION

To the best of our knowledge, this is the first study to show that protein kinase C positively regulates aquaporin-1 channels. Furthermore, we provide data demonstrating that this new pathway of AQP1 regulation is independent of the previously described cyclic nucleotide pathway.

Molecular Basis of AQP1 Regulation by Protein Kinase C—A parallel effect of protein kinase C on ion currents and water permeability mediated by aquaporin-1 channels was observed in this study. However, technically it is not feasible to measure both features of AQP1 function simultaneously. Furthermore, measurements of water permeability are limited by the mechanical robustness of the cells that do not tolerate excessive volume increases. Because the measurement of ion currents poses considerably less methodological limitations, the respective voltage clamp experiments were used for additional con-
firmatory experiments and for a more subtle analysis of the underlying molecular mechanisms.

Several lines of evidence are provided that protein kinase C regulates aquaporin-1 channel function. First, PKC activators OAG and PMA induced an increase of AQP1 function with a time course that is typical for kinase-dependent regulation in this expression system (13, 14, 26). This effect does not involve endogenous water channels or ion channels of Xenopus oocytes, because it was not observed in noninjected oocytes. Second, the effect could be suppressed by co-application of specific PKC inhibitor chelerythrine. Third, inactivation of the PKC consensus sites of AQP1 almost completely abolished the observed regulation. Interestingly, inactivation of either one of the two consensus sites attenuated the regulation to a relative current increase that was approximately in the middle in between the relative effects in wild type channels and in AQP1 ΔPKC channels. Hence, the regulatory effects of the two phosphorylation sites appear to be independent of each other with each site conferring approximately half of the total effect. Finally, in vitro phosphorylation assays using synthesized peptides demonstrated that peptides corresponding to the wild type domains surrounding Thr157 and Thr239 are phosphorylated by PKC, whereas homologous peptides corresponding to the T157A and T239A mutants are not. Together with the functional data, the phosphorylation data provide complementary evidence arguing for a major role for both PKC phosphorylation sites in the mediation of the observed effects.

AQP1 Activation via the PKC Pathway and via the Cyclic Nucleotide Pathway—To date, little attention has been paid to the short term regulation of AQP1. So far, only cyclic nucleotide (CN) signaling pathways have been conclusively shown to modulate AQP1 function; cGMP and also indirectly cAMP induce an increase of water permeability and give rise to cationic currents (2, 3). This effect could be reproduced in AQP1 wild type channels in this study. However, the molecular mechanism underlying CN regulation of AQP1 has not been fully elucidated yet because AQP1 does not exhibit a complete CN-binding domain and no cAMP-dependent protein kinase consensus sites (4, 29).

In this study, we observed a similar pattern with protein kinase C, i.e. an increase of both water permeability and ionic currents. We were therefore interested in obtaining information about potential links between these two pathways. Hence, AQP1 ΔPKC channels with almost completely abolished PKC-dependent regulation were exposed to forskolin and IBMX that induce an increase of intracellular CN levels. Those channels exhibited a marked current increase that was nearly identical to that of AQP1 wild type channels. We therefore conclude that significant cross-talk between those two pathways is unlikely and that both pathways use different molecular mechanisms to regulate AQP1 function.

Potential Physiological Implications—Traditionally, AQP1 has been classified as constitutively available pore maintaining constant water permeability (1). Its regulation has mainly been investigated on the transcriptional level with experimental evidence for an up-regulation by corticosteroids and by hypertonic stimuli via respective promoters (5). In this study, we have shown a pronounced regulation of AQP1 function by the PKC system. PKC signaling has been implicated in various physiological processes that recently have also been linked to AQP1 such as endothelial permeability, angiogenesis, and urine concentration (17–24). Notably, PKC promotes angiogenesis, and increased AQP1 function has been shown to be a determinant of endothelial cell migration and subsequent angiogenesis (7–9, 20–22). It is well documented that PKC signaling increases endothelial permeability and modulates urine concentration, both of which may involve an increase of AQP1-mediated water permeation (17–20, 23, 24). Hence, it is plausible to hypothesize that an increase of AQP1 function may also contribute to the stimulation of those processes by PKC.

AQP1 is one of several aquaporins that have been shown to conduct ion currents in addition to water (30, 31). However, the physiological role of those currents has remained a matter of debate because of the small ratio of ionic conductance to water permeation (32, 33). It has been suggested that aquaporin-mediated ion flow may be involved in the maintenance of ionic and osmotic balance of AQP1-expressing tissues (31), possibly comparable with the function ascribed to water-specific AQP4 channels in neuroglia (34). Recently, a related function of AQP1 water and ion transport has been shown in choroid plexus epithelium, thereby proving for the first time a physiological function of AQP1-mediated currents in native tissue (4). Notably, both the CN pathway (2, 3) and the PKC pathway described in this study induce a parallel increase of both ionic conductance and water permeability.

Conclusions—In summary, this study provides experimental evidence that protein kinase C positively regulates aquaporin-1 channels with a predominant role of both PKC phosphorylation sites at Thr157 and Thr239. This new pathway of AQP1 regulation is independent of the previously described cyclic nucleotide pathway. Activation of AQP1 may contribute to the PKC-dependent stimulation of physiological processes that are modulated by AQP1 such as endothelial permeability, angiogenesis, and urine concentration.

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REFERENCES
1. Agre, P., King, L. S., Yasui, M., Guggino, W. B., Ottersen, O. P., Fujiyoshi, Y., Engel, A., and Nielsen, S. (2002) J. Physiol. 542, 3–16
2. Anthony, T. L., Brooks, H. L., Boassa, D., Leonov, S., Yanochko, G. M., Regan, J. W., and Yool, A. W. (2000) Mol. Pharmacol. 57, 576–588
3. Yool, A. J., Stamer, D., and Regan, I. W. (1996) Science 273, 1216–1218
4. Boassa, D., Stamer, W. D., and Yool, A. J. (2006) J. Neurosci. 26, 7811–7819
5. Devuyst, O., Ni, J., and Verbavatz, J. (2005) Biol. Cell 97, 667–673
6. Ni, J., Verbavatz, J. M., Rippe, A., Boisde, I., Moulin, P., Rippe, B., Verkman, A. S., and Devuyst, O. (2006) Kidney Int. 69, 1518–1525
7. Sadoun, S., Papadopoulos, M. C., Hara-Chikuma, M., and Verkman, A. S. (2005) Nature 434, 786–791
8. Hara-Chikuma, M., and Verkman, A. S. (2006) J. Am. Soc. Nephrol. 17, 39–45
9. Belge, H., and Devuyst, O. (2006) Nephrol. Dial. Transplant. 21, 2069–2071

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10. Verkman, A. S. (2002) *J. Cell Sci.* **118**, 3225–3232
11. Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002) *Nature* **415**, 206–212
12. Nishizuka, Y. (1995) *FASEB J.* **9**, 484–496
13. Karle, C. A., Zitron, E., Zhang, W., Wendt-Nordahl, G., Kathöfer, S., Thomas, D., Gut, B., Scholz, E., Vahl, C. F., Katus, H. A., and Kiehn, J. (2002) *Circulation* **106**, 1493–1499
14. Kiesecker, C., Zitron, E., Scherer, D., Lueck, S., Bloehs, R., Scholz, E. P., Pirot, M., Kathöfer, S., Thomas, D., Kreye, V. A., Kiehn, J., Borst, M. M., Katus, H. A., Schoels, W., and Karle, C. A. (2006) *J. Mol. Med.* **84**, 46–56
15. Zelenina, M., Zelenin, S., Bondar, A. A., Brismar, H., and Aperia, A. (2002) *Am. J. Physiol.* **283**, F309–F318
16. Han, Z., Wax, M. B., and Patil, R. V. (1998) *J. Biol. Chem.* **273**, 6001–6004
17. Chang, Y. S., Munn, L. L., Hillsley, M. V., Dull, R. O., Yuan, J., Lakshminarayanan, S., Gardner, T. W., Jain, R. K., and Tarbell, J. M. (2000) *Microvasc. Res.* **59**, 265–277
18. Siflinger-Birnboim, A., and Johnson, A. (2003) *Am. J. Physiol.* **284**, L1435–L1451
19. Dang, L., Seale, J. P., and Qu, X. (2005) *Clin. Exp. Pharmacol. Physiol.* **32**, 771–776
20. Bokhari, S. M., Zhou, L., Karasek, M. A., Paturi, S. G., and Chaudhuri, V. (2006) *J. Investig. Dermatol.* **126**, 460–467
21. Wang, A., Nomura, M., Patan, S., and Ware, J. A. (2002) *Circ. Res.* **90**, 609–616
22. Ware, J. A., and Simons, M. (1999) *Angiogenesis and Cardiovascular Disease*, pp. 30–59, Oxford University Press, Oxford
23. Yao, L., Huang, D. Y., Pfaff, I. L., Nie, X., Leitges, M., and Vallon, V. (2004) *Am. J. Physiol.* **287**, F299–F304
24. Garcia, N. H., and Garvin, J. L. (1994) *J. Clin. Invest.* **93**, 2572–2577
25. Barish, M. E. (1983) *J. Physiol.* **342**, 309–325
26. Zitron, E., Kiesecker, C., Lück, S., Kathöfer, S., Thomas, D., Kreye, V., Kiehn, J., Katus, H. A., Schoels, W., and Karle, C. A. (2004) *Cardiovasc. Res.* **63**, 520–527
27. Zitron, E., Scholz, E. P., Owen, R., Luck, S., Kiesecker, C., Thomas, D., Kathöfer, S., Nirooamand, F., Kiehn, J., Kreye, V. A., Katus, H. A., Schoels, W., and Karle, C. A. (2005) *Circulation* **111**, 835–838
28. Witchel, H. J., Milnes, J. T., Mitcheson, J. S., and Hancox, J. C. (2002) *J. Pharmacol. Toxicol. Methods* **48**, 65–80
29. Boassa, D., and Yool, A. J. (2003) *BMC Physiol.* **3**, 12
30. Yu, J., Yool, A. J., Schulten, K., and Tajkhorshid, E. (2006) *Structure* **14**, 1411–1423
31. Yool, A. J., and Weinstein, A. M. (2002) *News Physiol. Sci.* **17**, 68–72
32. Tsunoda, S., Wiesner, B., Lorenz, D., Rosenthal, W., and Pohl, P. (2004) *J. Biol. Chem.* **279**, 11364–11367
33. Saparov, S. M., Kozono, D., Rothe, U., Agre, P., and Pohl, P. (2001) *J. Biol. Chem.* **276**, 31515–31520
34. Amiry-Moghaddam, M., Williamson, A., Palomba, M., Eid, T., de Lanerolle, N. C., Nagelhus, E. A., Adams, M. E., Froehner, S. C., Agre, P., and Ottersen, O. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13615–13620