AQP4 plasma membrane trafficking or channel gating is not significantly modulated by phosphorylation at COOH-terminal serine residues

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Submitted 13 June 2014; accepted in final form 11 September 2014

AQP4 plasma membrane trafficking or channel gating is not significantly modulated by phosphorylation at COOH-terminal serine residues. Am J Physiol Cell Physiol 307: C957–C965, 2014. First published September 17, 2014; doi:10.1152/ajpcell.00182.2014.—Aquaporin 4 (AQP4) is the predominant water channel in the mammalian brain and is mainly expressed in the perivascular glial endfeet at the brain-blood interface. AQP4 serves as a water entry site during brain edema formation, and regulation of AQP4 may therefore be of therapeutic interest. Phosphorylation of aquaporins can regulate plasma membrane localization and, possibly, the unit water permeability via gating of the AQP channel itself. In vivo phosphorylation of six serine residues in the COOH terminus of AQP4 has been detected by mass spectrometry: Ser111, Ser180, Ser315, Ser316, Ser221, and Ser322. To address the role of these phosphorylation sites for AQP4 function, serine-to-alanine mutants were created to abolish the phosphorylation sites. All mutants were detected at the plasma membrane of transfected C6 cells, with the fraction of the total cellular AQP4 expressed at the plasma membrane of transfected C6 cells being similar between the wild-type (WT) and mutant forms of AQP4. Activation of protein kinases A, C, and G in primary astrocytic cultures did not affect the plasma membrane abundance of AQP4. The unit water permeability was determined for the mutant AQP4s upon heterologous expression in Xenopus laevis oocytes (along with serine-to-aspartate mutants of the same residues to mimic a phosphorylation). None of the mutant AQP4 constructs displayed alterations in the unit water permeability. Thus phosphorylation of six different serine residues in the COOH terminus of AQP4 appears not to be required for proper plasma membrane localization of AQP4 or to act as a molecular switch to gate the water channel.

water permeability; astrocyte volume regulation; aquaporin regulation

BRAIN EDEMA occurs as excess fluid transfers from the blood to the brain parenchyma. An ensuing swelling of tissue volume results in increased intracranial pressure, which may in severe cases be fatal (45). The molecular mechanism(s) and signaling pathways underlying the unwarranted cerebral fluid accumulation are not fully identified and may well differ depending on the pathological stimulus giving rise to the edema. Aquaporin 4 (AQP4) is the predominant water channel in the brain (1). It is localized in the perivascular glial endfeet, the ependymal cell lining, and osmosensing areas such as the supraoptic nucleus and subfornical organ (30). AQP4 exists as three functional isoforms (23) with the shortest isoform, M23, as the most abundant isoform in the brain (29). M23 is the molecular substrate for the characteristic orthogonal arrays of particles observed at the astrocytic endfeet (36). Due to the location of AQP4 at the blood-brain interface and the altered outcome of AQP4 knock-out mice following experimentally inflicted brain edema formation, AQP4 has been proposed to be involved in the formation of brain edema (reviewed in Ref. 46). Dynamic regulation of AQP4, possibly via protein phosphorylation, may therefore be a possible molecular mechanism instigating brain edema formation. Phosphorylation of proteins, as a reversible posttranslational modification, can regulate trafficking, conformational changes, functional activity, molecular association, and/or localization of proteins. The amino acid sequence of AQP4 contains consensus sequences for several protein kinases (PK), such as PKA, PKC, PKG, Ca2+/calmodulin-dependent protein kinase II, and casein kinase II (9). A consensus sequence for a protein kinase represents a sequence of amino acids that are amenable to phosphorylation by this kinase and may or may not be phosphorylated in vivo. Phosphorylation-dependent dynamic regulation of AQP4 can be achieved by changes in levels of AQP4 membrane expression via altered trafficking to and from the plasma membrane or by changes in the water permeability of AQP4 already present in the membrane, i.e., “gating.” PKG and PKA have been proposed to phosphorylate Ser111 in AQP4 and thereby provoke a gating event leading to increased osmotic water permeability through AQP4 (10, 38) whereas a PKC-dependent phosphorylation of Ser180 was reported to produce a closure of the gate (47). These gating events have, however, been challenged by the crystal structure of AQP4 (11, 40), the lack of evidence in favor of in vivo phosphorylation of Ser111 (2), and functional studies as well as molecular dynamics simulations providing evidence against phosphorylation-dependent gating of AQP4 via Ser111 and Ser180 (2, 22, 24, 37).

In vivo phosphorylation of Ser111 or Ser180 has not been detected by mass spectrometry, although a range of other protein phosphorylation sites in the COOH terminus of AQP4 have been detected by this experimental approach: six serine residues in the COOH terminus of AQP4 (Ser111, Ser180, Ser315, Ser316, Ser321, and Ser322; Table 1) (12–14, 19, 41–43). The physiological impact of phosphorylation of these residues remains unresolved, and accordingly, we aimed to determine the functional importance of the serine phosphorylation sites detected in the COOH terminus of AQP4. Regulation of AQP4 membrane trafficking was determined in primary cultures of astrocytes as well as by heterologous expression of mutant forms of AQP4 in a C6 glioma cell line whereas putative gating events were determined upon expression of these constructs in Xenopus laevis oocytes.

MATERIALS AND METHODS

Molecular biology. Rat AQP4.M23 was subcloned into the oocyte expression vector pXOOM, linearized downstream from the poly-A

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Table 1. COOH-terminal serine phosphorylation sites in AQP4

<table>
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<th>Residue</th>
<th>Functional Studies (Ref. No.)</th>
<th>Mass Spectrometry Studies (Ref. No.)</th>
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<tr>
<td>Ser276</td>
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<td>13, 14, 19, 42</td>
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First column contains amino acid and number (kept according to that of the M1 isoform). The second column contains references on functional studies on these residues, and the third column contains references for mass spectrometry studies in which these phospho-sites were detected.

segment, and in vitro transcribed using T7 mMessage Machine (Ambion, Austin, TX). MEGAClear (Ambion, Austin, TX) was used to extract cRNA, which was microinjected into defolliculated Xenopus laevis oocytes. Mutations in AQP4 were introduced with Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, CA) and verified with DNA sequencing. Numbering of the AQP4 amino acids is for convenience kept according to that of AQP4.M1.

Oocyte preparation. Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI), National Center for Scientific Research (France), or EcoCyte BioScience (Germany). All animal protocols comply with the European Community guidelines for the use of experimental animals and were approved and performed under a license issued for the use of experimental animals by the Danish Ministry of Justice (Dyreforsøgstilsynet). Oocytes were surgically removed from anesthetized frogs, and the follicular membrane was removed by incubation in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4, 182 mOsM) containing 10 mg/ml collagenase (Type I, Worthington, NJ) and 1 mg/ml trypsin inhibitor (Sigma, Denmark) as previously described (7). After microinjection of cRNA encoding AQP4 (25 ng/oocyte), the oocytes were kept in Kulori medium at 19°C for 3–4 days prior to experiments.

Oocyte volume measurements. The experimental setup for measuring water permeability of oocytes has been described in detail previously (48). Briefly, the oocyte was placed in a small chamber with a glass bottom and perfused with a control solution at room temperature (RT) (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4). The oocyte was viewed from below via a long-distance objective, and oocyte images were captured continuously at a rate of 25 images/s. To determine the water permeability, the oocytes were challenged with a hypertonic solution (control solution + 300 mOsm mannitol), and the water permeability was calculated as

\[ J_p = \frac{J_v}{A \cdot \Delta m \cdot V_w} \]

where \( J_v \) is the water flux during the osmotic challenge, \( A \) is the true membrane surface area (~9 times the apparent area due to membrane folding [46]), \( \Delta m \) is the osmotic change, \( V_w \) is the partial molal volume of water (18 cm³/mol), and \( J_p \) is the water permeability (given in units of cm/s).

Oocyte immunocytochemistry and confocal laser scanning microscopy. The oocytes (n = 5 per construct per experiment) were fixed for 1 h in 3% paraformaldehyde (PFA) in control solution and subsequently stored at 4°C in control solution containing 0.3% PFA. Preparation of the oocytes for imaging has been described previously (25). Briefly, the oocytes were dehydrated, paraffin embedded, and cut into sections (2 μm) on a Leica RM 2126 microtome. The sections were immunostained with a rabbit polyclonal anti-AQP4 antibody 1:5,000 (Alomone Laboratories) followed by an Alexa 488-conjugated secondary antibody 1:1,000 for visualization (DAR, Invitrogen). Leica TCS SL confocal microscope with a HCX PL APO ×40 oil objective lens and Leica confocal software were used for imaging of the oocytes. Image semiquantification and validation were carried out as previously described (25).

Primary cultures of astrocytes. Astrocytes were prepared as previously described (18). Briefly, cortical rat astrocytes were cultured from dissected cerebral cortices of postnatal day 7–8 rat pups (Sprague-Dawley, Taconic, Denmark). The dissected cortices were dissociated mechanically by passing the tissue through an 80-μm nylon sieve into Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) containing an additional 6 mM d-glucose, 2.5 mM l-glutamine, 26.2 mM NaHCO₃, 100,000 IU/l of penicillin, and 20% fetal bovine serum (Biological Industries). Cells were plated in six-well tissue culture plates, and the fetal bovine serum concentration was sequentially reduced to 15% and 10% on the second and third week of culture. The astrocytes were kept at 37°C with an atmosphere of 5% CO₂ and were used for experiments 3–4 wk after plating.

Immunocytochemistry and confocal imaging. C6 cells were seeded in six-well plates containing coverslips (50% confluenence) and transiently transfected with wild-type (WT) AQP4.M23 or mutant AQP4-1 (1 h analysis) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The C6 cells were fixed with 2% paraformaldehyde (15 min) 48 h after transfection. Astrocytes were split after 3 wk in culture (to obtain single cells) and fixed 24 h later in 2% paraformaldehyde (15 min). The cells were incubated 10 min at room temperature (RT) with wheat germ agglutinin (WGA), Alexa Fluor 555 Conjugate (5.0 μg/ml; Invitrogen), washed with PBS and permeabilized in PBS with 0.2% BSA and 0.2% Triton X-100. AQP4 was visualized with a rabbit polyclonal anti-AQP4 antibody, 1:500 (Alomone Laboratories) and a goat anti-rabbit 488 Alexa-conjugated secondary antibody, 1:500 (Invitrogen). The cells were mounted in Prolong Gold mounting medium (Invitrogen), and imaged in a laser scanning microscope (Zeiss LSM 710, Carl Zeiss MicroImaging, Jena, Germany) using a 63× 1.4 NA oil objective.

Biotinylation assay. C6 cells were seeded in six-well plates and transfected as described above. Cell surface biotinylation was performed 48 h after transfection of C6 cells. The primary cultures of astrocytes were used 3–4 wk after plating and were incubated with either vehicle (0.1% DMSO) or different protein kinase activators for 1 h analyzed by the experimental procedure. The biotinylation was performed as previously described (26): the cells were placed on ice, washed three times with ice-cold PBS-CM (PBS with addition of 1 mM CaCl₂, 0.1 mM MgCl₂, pH 7.5), and incubated for 45 min at 4°C in ice-cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl₂, 125 mM NaCl, pH 8.9) containing sulfo-NHS-SS-biotin (1 mg/ml final concentration, Thermo Scientific, Rockford, IL). The cells were washed with ice-cold quenching buffer (PBS-CM, 50 mM Tris-HCl, pH 8.0) followed by wash in PBS-CM. Cells were scraped and centrifuged at 4,000 g for 5 min at 4°C, and the pellets were resuspended in 500 μl lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 μg/ml leupeptin, 200 μg/ml pepstatin). Samples were sonicated on ice prior to centrifugation at 10,000 g for 10 min at 4°C. A fraction of the supernatant was retained for total AQP4 estimates. The remaining supernatant was transferred to spin columns containing Immobilized NeutrAvidin gel slurry (Thermo Scientific) and incubated for 10 min at RT. After extensive washing, samples were eluted in elution buffer (50 mM Ultrapure Tris, 6% glycerol, 50 mM SDS, bromophenol blue, 12 mg/ml DTT) and analyzed on immunoblots with a rabbit polyclonal anti-AQP4 antibody 1:1,000 (Alomone Laboratories), a rabbit polyclonal anti-ERK antibody 1:1,000 (Cell Signaling), or a rabbit polyclonal anti-proteasome 20S C2 subunit antibody (Abcam). A horseradish peroxidase-conjugated secondary antibody (P448, Dako) was used at a concentration of 1:3,000. Visualization was obtained with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) and imaged with BioSpectrum AC imaging system (UVG, Upland, CA). Data were obtained from four independent
experiments for C6 cells and from three independent astrocyte preparations.

**Chemicals.** Leupeptin (1 μg/ml), pefabloc (200 μg/ml), vasopressin (1 μM), and 8-Br-cGMP (100 μM) were all dissolved in water. 8-Br-cAMP (300 μM) was dissolved in water containing 94 μM Tris base, and phorbol 12-myristate 13-acetate (PMA, 200 nM) was dissolved in DMSO. All were obtained from Sigma Aldrich.

**Statistics.** Data are presented as means ± SE. ANOVA followed by Dunnett's multiple comparisons tests were used for the statistical analysis. A probability level of *P* < 0.05 was considered statistically significant.

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**RESULTS**

Phosphorylation of serine residues in the COOH terminus of AQP4 is not required for trafficking of AQP4 to the plasma membrane. In vivo phosphorylation of the COOH terminus of AQP4 has been detected by mass spectrometry, and the phosphorylated serine residues in the primary amino acid sequence are depicted in the topology diagram of AQP4 in Fig. 1 (references contained in Table 1). Analysis of the conservation of these sites (and additional suggested phosphorylation sites at Ser^111^ and Ser^180^ among species was performed as an initial indicator of the functional importance of each site. Using the bioinformatics software “CPhos” (50), all of the analyzed phospho-sites and surrounding motifs were to a large extent conserved across the species examined (Fig. 2). The present study focuses on the functional requirement for phosphorylation of these serine residues. To resolve the role of these phospho-sites in membrane trafficking, we initially employed a mutational strategy followed by heterologous expression in a mammalian cell system devoid of endogenous AQP4. The individual serines were replaced with alanines by site-directed mutagenesis to prevent phosphorylation at the given residue. The phosphorylation sites containing two neighboring serines were generated as double mutants (S315.316A and S321.322A). To determine if phosphorylation of these residues is required for correct targeting to the plasma membrane, the WT AQP4 and the four alanine mutants (S276A, S285A, S315.316A, and S321.322A) were heterologously expressed in rat C6 cells, a glioma cell line (of astrocytic origin) devoid of endogenous AQP4 expression (31). To visualize the membrane compartment in the subsequent immunocytochemistry, the cells were stained with the glycoprotein-binding lectin WGA prior to permeabilization of the cells and staining for AQP4. The representative confocal ortho-images of Z-stacks illustrate robust expression of WT AQP4 and all four AQP4 mutants at the plasma membrane of the C6 cells.
No AQP4 staining was detected in the native cells (Fig. 3). From the X-Z and Y-Z images it is apparent that AQP4 (green) is localized at the cell membrane (marked in red with WGA staining). The images obtained with immunocytochemistry thus demonstrate that phosphorylation of the respective amino acid residues is not required for localization of AQP4 to the plasma membrane of C6 cells.

Prevention of phosphorylation of serine residues in the COOH terminus does not quantitatively alter the expression of AQP4 at the plasma membrane. To obtain a semiquantitative measure of the plasma membrane expression of AQP4 vs. its mutant constructs, we employed semiquantitative biotinylation of AQP4-expressing C6 cells. To ensure selective labeling of AQP4 in the plasma membrane, we initially verified that biotin access to the cytosolic compartment was prevented under the experimental conditions. C6 cells were surface-biotinylated followed by purification of the biotinylated fraction. This biotin-purified sample was, along with a control sample containing total cell lysate, separated by SDS-polyacrylamide gel electrophoresis. Subsequent Western blotting, with an antibody directed against the cytoplasmic protein kinase ERK, detected the presence of ERK in the sample containing total cell lysate (Fig. 4A, left panel) whereas no ERK immunoreactivity was detected in the biotin-purified samples, which should exclusively contain the plasma membrane fraction (Fig. 4A, right panel). The biotinylated fraction, obtained with the present

Fig. 4. The amount of AQP4 at the plasma membrane relative to the total amount is not changed by COOH-terminal phosphorylation. A: representative Western blots of total C6 cell lysate samples (left panel) or biotin-purified samples (right panel) probed with anti-ERK antibody (n = 4). The C6 cells were either nontransfected or transiently transfected with WT AQP4. Two isoforms of the cytoplasmic protein ERK is detected [ERK1 (44 kDa) and ERK2 (42 kDa)] in the total cell lysate samples while no ERK was detected in the biotin-purified samples containing the plasma membrane fraction of the cells. B: representative Western blots of total C6 cell lysate samples (bottom panel) or biotin-purified samples (top panel) probed with anti-AQP4 antibody. The C6 cells were either nontransfected or transiently transfected with WT AQP4, AQP4.S276A, AQP4.S285A, AQP4.S315.316A, or AQP4.S321.322A. C: ratio of AQP4 expressed at the membrane relative to the total amount for the 4 mutants normalized to the WT, n = 4. ANOVA followed by Dunnett’s multiple comparison test was used as statistical test; ns, not significant.
NO PHOSPHORYLATION-DEPENDENT TRAFFICKING AND GATING OF AQP4

Experimental design, is thus suitable for determination of the fraction of AQP4 expressed in the plasma membrane. C6 cells expressing AQP4 or its mutant versions were surface biotinylated, the biotin-containing membrane fraction purified, and the samples exposed to Western blotting with an antibody directed toward AQP4. A representative Western blot of the biotin-purified fraction of AQP4-expressing C6 cells is illustrated in Fig. 4B, top panel. All AQP4 constructs were expressed at the cell membrane as evident by the presence of AQP4 labeling in all lanes containing samples from AQP4-expressing C6 cells. In Fig. 4B, bottom panel, Western blotting of total cell lysate illustrates the total AQP4 content (in intra- and extracellular compartments) in parallel cell samples. The ratio between the density of the biotinylated sample and the total sample signifies the fraction of AQP4 present at the plasma membrane. This fraction was normalized to that of the WT AQP4 and averaged across four identical experiments (Fig. 4C). Although the expression level of the different constructs varied, there was no significant difference between fractional plasma membrane expression of WT AQP4 and the four mutants (in % of AQP4 WT: 89.9 ± 9.0 for AQP4.S276A, 100.8 ± 4.9 for AQP4.S285A, 90.6 ± 1.9 for AQP4.S315.316A, 97.0 ± 36.7 for AQP4.S321.322A, n = 4, summarized in Fig. 4C). It appears that prevention of phosphorylation at Ser276, Ser285, Ser315/316, and Ser321/322 did not interfere with proper targeting of AQP4 to the plasma membrane.

Activation of PKA, PKC, and PKG does not affect the membrane expression of AQP4. AQP4 is natively expressed in astrocytes and predominantly, although not exclusively, located at the plasma membrane of primary cultures of rat astrocytes (Fig. 5A). To determine the effect of activation of PKA, PKC, and PKG on AQP4 trafficking in primary cultures of rat astrocytes, we performed semiquantitative surface biotinylation of AQP4 in these native cultures. To ensure that biotin did not gain access to the cytosolic compartment (and therefore cytosolic AQP4) during the experimental procedure, we initially verified the absence of the cytosolic protein proteasome 20S C2 subunit in the biotin-purified fraction of astrocytes (Fig. 5B). PKA (32, 39, 49), PKC (27, 34, 44), and PKG (3, 33) were activated in cultured astrocytes by exposure to their respective membrane-permeable activators for 60 min (300 μM 8-Br-cAMP for PKA, 200 nM PMA for PKC, and 100 μM 8-Br-cGMP for PKG). At the termination of the experimental procedure, the astrocytes were exposed to biotin, the biotinylated fraction purified, and the samples exposed to Western blotting with an antibody directed against AQP4 (see Fig. 5C, top panel for representative Western blot). The relative plasma membrane fraction of AQP4 was determined as the densitometry of the biotinylated samples divided by the densitometry of the samples exposed to Western blotting with an antibody directed against AQP4 (see Fig. 5B, bottom panel for representative Western blot). The relative plasma membrane expression of WT AQP4 and the four mutants (in % of AQP4 expressed at the membrane relative to the total amount for the 4 treatments normalized to the control, n = 6) of total primary astrocytic cell lysate samples (left lane) or biotin-purified sample (right lane) probed with anti-proteasome 20S C2 subunit antibody. The cytoplasmic protein proteasome 20S C2 subunit was detected in the total cell lysate samples while no proteasome 20S C2 subunit was detected in the biotin-purified samples containing the plasma membrane fraction of the cells. C: representative Western blots of biotin-purified samples (top panel) or total primary astrocyte cell lysate samples (bottom panel) probed with an anti-AQP4 antibody. The astrocytes were either vehicle treated (0.1% DMSO, control) or treated with a PKA activator (8-Br-cAMP, 300 μM), a PKC activator (PMA, 200 μM), a PKG activator (8-Br-cGMP, 100 μM), or vasopressin (1 μM). D: ratio of AQP4 expressed at the membrane relative to the total amount for the 4 treatments normalized to the control, n = 6. ANOVA followed by Dunnett’s multiple comparison test was used as statistical test; ns, not significant.
AQP4 in the total cell lysate samples from the same experiment (see Fig. 5C, bottom panel for representative Western blot). The fractions of AQP4 in the plasma membrane were normalized to that of the control astrocytes and averaged across six identical experiments from three batches of astrocytes (Fig. 5D). Activation of PKA, PKC, or PKG did not significantly change the membrane fraction of AQP4 (in % of control: 97.2 ± 10.0 for 8-Br-cAMP, 93.3 ± 7.6 for PMA, and 90.8 ± 9.0 for 8-Br-cGMP, n = 6, summarized in Fig. 5D). In addition, we exposed the cultured astrocytes to vasopressin prior to surface-biotinylation to evaluate a putative vasopressin receptor 1a (V1aR)-mediated internalization of AQP4. Vasopressin did not produce internalization of AQP4 in cultured astrocytes (membrane fraction of 98.3 ± 11.4% of that of the control condition, n = 6; Fig. 5, C and D). Thus activation of the protein kinases tested in the present study did not alter the plasma membrane abundance of AQP4 in astrocytes.

Phosphorylation of serine residues in the COOH terminus of AQP4 is not involved in gating of the channel. Phosphorylation of AQP4 could potentially affect function of AQP4 already present in the plasma membrane by introducing a gating event of the channel, thus moderating water transport capacity (10). To determine the relative unit water permeability of the mutant AQP4 constructs in comparison to their WT counterpart, we expressed the constructs in Xenopus oocytes. Due to the low intrinsic water permeability of the native oocyte membrane and the favorable oocyte surface-to-volume ratio, this expression system is well-suited for quantitative osmotic water permeability measurements of heterologously expressed proteins. The osmotic water permeability of the oocytes was evaluated with a sensitive camera upon an abrupt challenge with a hypertonic test solution containing an additional 20 mM mannitol (20 mOsm). The water permeability of the oocytes increased ~15-fold upon expression of WT AQP4 [compare 1.62 ± 0.08 × 10⁻³ cm/s (n = 30) with 0.10 ± 0.01 × 10⁻³ cm/s (n = 10)].

To determine a putative phosphorylation-dependent gating of AQP4 via the COOH-terminal serine residues, we generated additional mutant constructs in which the serines were replaced with aspartate. The negatively charged aspartate may mimic a phosphorylated residue (20). The water permeability was determined in oocytes expressing WT AQP4, the four mutant constructs in which the serines were replaced with alanine to prevent phosphorylation (S276A, S285A, S315,316A, S321,322A), and the four mutant constructs in which the serines were replaced by aspartate to mimic phosphorylation (S276D, S285D, S315,316D, S321,322D) (n = 3 oocytes expressing each construct per experiment). All mutant constructs gave rise to functional expression of AQP4, as seen from the representative volume traces shown in Fig. 6A. As membrane expression levels of AQP4 may vary between batches of oocytes and between constructs, the membrane abundance of AQP4 was semiquantified within each day-matched batch of oocytes (n = 5 of each construct per experiment) by immunocytochemistry.

**Fig. 6.** No change in the relative unit water permeability by mutation of Ser²⁷⁶, Ser²⁸⁵, Ser³¹⁵/³¹⁶, or Ser³²¹/³²². A: representative volume traces from oocytes challenged with a hyperosmotic gradient of 20 mOsm mannitol as marked by the black bar above the trace as a function of time; see inset for axes labeling (n = 3 oocytes per experiment, 5 experiments). Each volume trace is paired with confocal laser scanning microscopy of an oocyte expressing the same construct after immunolabeling with anti-AQP4 antibodies (n = 5 oocytes per experiment, 5 experiments). The oocytes were either un.injected or expressed WT AQP4, AQP4.S276A, AQP4.S276D, AQP4.S285A, AQP4.S285D, AQP4.S315,316A, AQP4.S315,316D, AQP4.S321,322A, or AQP4.S321,322D. B: the relative unit water permeability was obtained by dividing the average water permeability with AQP4 membrane abundance (oocyte plasma membrane fluorescent counts) from the same experiment and normalized to that of WT AQP4 (averaged across 5 experiments). ANOVA followed by Dunnett’s multiple comparison test was used as statistical test; ns, not significant.
with an antibody directed toward AQP4. We have previously validated this quantification method by comparison with immunoblotting of the purified membrane fraction of AQP4-expressing oocytes (7, 25). Representative confocal images of each construct is shown adjacent to its volume trace in Fig. 6A. Based on the water permeability and the membrane abundance of each individual construct, the relative unit water permeability was obtained for each batch of oocytes by normalization to that of the WT AQP4 and averaged across five experimental batches of oocytes (Fig. 6B). Although some variability was observed between the WT and the mutant AQP4s, there was no statistically significant difference between their relative unit water permeability as assessed with ANOVA followed by Dunnett’s multiple comparison test (unit water permeability in % of WT: 95.1 ± 10.9 for AQP4.S276A, 92.2 ± 14.7 for AQP4.S276D, 113.7 ± 17.0 for AQP4.S285A, 86.1 ± 15.2 for AQP4.S285D, 77.6 ± 5.6 for AQP4.S315.316A, 77.6 ± 4.9 for AQP4.S315.316D, 109.5 ± 11.9 for AQP4.S321.322A, 77.9 ± 9.0 for AQP4.S321.322D, n = 5). It thus appears that the water pore of AQP4 is open independently of the phosphorylation state of these serines in the COOH terminus of AQP4.

Prevention of phosphorylation of all six C-terminal serine residues does not alter the membrane targeting nor the water permeability of AQP4. Phosphorylation sites can be functionally redundant in protein regulation and would, as such, disguise an effect of individual mutations to alanine. To determine the effect of combined elimination of all COOH-terminal serine phosphorylation sites, we created an AQP4 mutant construct in which all six serine residues were mutated to alanine. The mutant version of AQP4 (AQP4.S276.285.315.316.321.322A) was heterologously expressed in rat C6 cells and AQP4 expression visualized with immunocytochemistry. The plasma membrane expression of the AQP4.S276.285.315.316.321.322A mutant (Fig. 7A) was similar to that of the WT AQP4 (compare with Fig. 3), suggesting that phosphorylation of the six COOH-terminal serine residues were not required for AQP4 plasma membrane localization. The water permeability of the same mutant was determined in Xenopus oocytes and the membrane abundance was semiquantified by immunocytochemistry (Fig. 7B). The relative unit water permeability of the AQP4.S276.285.315.316.321.322A mutant expressed in Xenopus oocytes was not significantly different from that of the WT AQP4 (105.2 ± 10.9% of AQP4 WT, n = 5; Fig. 7C). Localization of AQP4 to the membrane and AQP4-dependent unit water permeability therefore do not seem to require phosphorylation of the six COOH-terminal serine residues alone or in combination.

**DISCUSSION**

In the present study we evaluated the role of known in vivo serine phosphorylation sites in the COOH terminus of AQP4 for localization to the cell membrane, kinase-dependent regulation of membrane abundance, and channel gating. Phosphorylation of Ser276, Ser285, Ser315, Ser316, Ser321, and Ser322 was not required for targeting of transiently transfected AQP4 to the plasma membrane of C6 cells. All transfected WT or mutant versions of AQP4 were localized to the plasma membrane in C6 cells, and large amounts of intracellular protein accumulation were not observed. Both confocal imaging and semiquantiitative biotinylation studies indicated that the fraction of AQP4 targeted to the membrane was similar for the mutants and WT AQP4. Thus phosphorylation of Ser276, Ser285, Ser315/316, and Ser321/322, alone or in combination, does not appear to be required for proper targeting of AQP4 to the plasma membrane of transiently transfected C6 cells. Notably, the S321.322A mutant was consistently detected at lower levels compared with WT AQP4 in the Western blots, although with a similar fractional membrane expression. It should be noted that the antibody recognizes an unknown epitope within the COOH-terminal of AQP4 (residues 249–323). It cannot be
ruled out that a given mutation changes the affinity for the antibody. However, since our experimental approach relies on relative membrane expression, our fractional data should not be influenced by differing antibody affinity. However, a similarly reduced expression level was previously observed in HEK293 cells expressing a COOH terminal (Ser^{231}, Ser^{322}, and Val^{323}) truncated version of AQP4 (28). The lower expression was due to a higher degradation rate, but in agreement with the present study, the COOH-terminal SSV was not essential for plasma membrane targeting of AQP4.

PKA, PKC, and PKG have all been proposed to phosphorylate AQP4 (10, 24, 38, 47) although the functional outcome of a putative phosphorylation is debated (2, 24). In cultured astrocytes, activation of PKA, PKC, and PKG by their respective membrane-permeable activators has consistently been demonstrated (3, 27, 32–34, 39, 44, 49). Nevertheless, activation of these kinases in cultured astrocytes, natively expressing AQP4, did not alter the fraction of AQP4 expressed at the membrane. These kinases therefore do not regulate plasma membrane abundance of AQP4 in the present experimental setting. As the majority of the astrocytic AQP4 was located at the plasma membrane at the onset of the experiment (this study and Ref. 35), a putative small kinase-dependent increase of AQP4 abundance in the plasma membrane could have been obscured in the present experimental approach. We previously demonstrated that in Xenopus oocytes, PKC activation (via activation of the vasopressin receptor V1aR) did not promote a gating event in AQP4 (24). In this heterologous expression system, however, a PKC-dependent internalization of AQP4 took place (24), an observation that was not paralleled in cultured astrocytes in the present study. Whether this discrepancy is due to differential expression levels of the vasopressin receptor in V1aR-expressing oocytes vs. astrocytes and/or cell-specific intracellular signaling machinery remains unresolved.

Phosphorylation-dependent gating of aquaporins has been suggested for both AQP2 and AQP4 (6, 8, 10, 16, 38) although subsequently questioned (2, 11, 17, 25, 37, 40). Removal or mimicking of individual COOH-terminal phosphorylation sites in AQP4 had no statistically significant effect on the unit water permeability of AQP4 and neither had removal of all six COOH-terminal phosphosites in combination. Phosphorylation of these specific serine residues thus appears to have no involvement in gating of AQP4.

Although our data indicate that serine phosphorylation in the COOH terminus of AQP4 is not required for gating of AQP4 or for proper membrane targeting of AQP4, our findings do not preclude phosphorylation-dependent dynamic regulation of AQP4 expression at the plasma membrane in a given cell type and/or by a specific kinase activation. Histamine was shown to induce internalization of AQP4 expressed in a gastric cell line via its activity on the G protein-coupled histamine receptor coupled to cAMP and PKA (4, 5). However, the internalization appeared to be PKA independent, and the phosphorylation of AQP4 occurred subsequently to the internalization of AQP4. Therefore phosphorylation of AQP4 by PKA (at an unidentified site) might be involved in retaining AQP4 in a vesicle-recycling compartment and thus in favoring recycling of AQP4 over degradation (5). In Madin-Darby canine kidney (MDCK) cells, casein kinase (CK) II phosphorylation of Ser^{276} had no effect on plasma membrane targeting or rate of internalization of AQP4 (21). Upon internalization, however, CKII-mediated phosphorylation of Ser^{276} appeared to enhance lysosomal targeting and degradation of AQP4 via increased interaction between AQP4 and the adaptor protein AP3 (21). In addition to the six serine residues phosphorylated in AQP4, Thr^{289} and Tyr^{277} have been detected in a phosphorylated state by mass spectrometry (14). Tyr^{277} has been proposed to be involved in clathrin-mediated endocytosis through interaction with the AP2 adaptor complex (21), although a requirement for phosphorylation of Tyr^{277} in endocytosis was not addressed. Thr^{289} was mutated to an alanine along with Ser^{276}, Ser^{285}, and Ser^{316} and this quadruple mutant was subsequently retained in the Golgi apparatus (15). Whether CKII-dependent phosphorylation of the COOH terminus of AQP4 is indeed necessary for Golgi transition (15) or whether this quadruple mutation could affect the protein folding to an extent to which it will be retained in Golgi simply due to misfolding remains to be fully elucidated.

In conclusion, we illustrate that COOH-terminal serine phosphorylation of AQP4 is not involved in channel gating, plasma membrane targeting, or membrane regulation of AQP4 expressed in primary cultures of astrocytes. COOH-terminal phosphorylation may rather, according to previously published data (5, 15, 21), be required for determination of the fate of AQP4 present in cytosolic compartments.

ACKNOWLEDGMENTS

We greatly value the technical assistance provided by C. G. Iversen, C. C. Goncalves Andersen, I.-M. Paulsen, and B. Kruse. We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen.

GRANTS

The project was funded by the Lundbeck Foundation (to N. MacAulay, M. Assentoft, and R. A. Fenton), the Danish Medical Research Council (to N. MacAulay and R. A. Fenton), and the Novo Nordisk Foundation (to N. MacAulay and R. A. Fenton).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


