Vasopressin-induced differential stimulation of AQP4 splice variants regulates the in-membrane assembly of orthogonal arrays

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Van Hoek AN, Bouley R, Lu YX, Silberstein C, Brown D, Wax MB, Patil RV. Vasopressin-induced differential stimulation of AQP4 splice variants regulates the in-membrane assembly of orthogonal arrays. Am J Physiol Renal Physiol 296: F1396–F1404, 2009. First published March 18, 2009; doi:10.1152/ajprenal.00018.2009.—Aquaporin-4 (AQP4) is a basolateral water channel in collecting duct principal cells and assembles into orthogonal array particles (OAPs), the size of which appears to depend on relative expression levels of AQP4 splice variants. Because the higher-order organization of AQP4 was perturbed by vasopressin in Brattleboro rats and phosphorylation sites have been identified on AQP4, we investigated whether vasopressin and forskolin (Fk) affect AQP4 assembly and/or expression in LLC-PK1 cells stably transfected with the AQP4 splice variant M23, which is responsible for formation of OAPs, and/or the splice variant M1, which does not form OAPs. Our data show that [lys8]-vasopressin (LVP) and Fk treatment led to differential increases in expression levels of M23-AQP4 and M1-AQP4 that varied as a function of incubation time. At early time points (day 1) expression of M1 was significantly stimulated (4.5-fold), over that of M23 (1.6-fold), but after 3 days the expression of M23 became predominant (4.1-fold) over that of M1 (1.9-fold). This pattern of stimulation was dependent on an intact AQP4 residue serine 111 and required protein synthesis. In cells expressing both M1 and M23 (M1/M23 ~ 1), with small sized OAPs at the membrane, the LVP/Fk-induced stimulation of M23 was modified and mimicked that of M1 when expressed alone, suggesting a dominant role for M1. In Brattleboro kidney inner medulla, an 8-day chronic exposure to the vasopressin agonist (dDAVP) led to reduction in M1 and a significant increase in M23 immunoblot staining (M1/M23 = 2/3 → 1/4). These results indicate that AQP4 organization and expression are regulated by vasopressin in vivo and in vitro and demonstrate that the dominant role for M1 is restricted to a one-to-one interaction between AQP4 splice variants that regulates the membrane expression of OAPs.

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THE WATER CHANNEL
AQUAPORIN-4 (AQP4) is a hydrophobic plasma membrane protein and functions as a water-selective channel facilitating osmotic water transport in several cell types, including kidney collecting duct principal cells (3, 6, 9, 30, 32). Unlike other water channels, two distinct isoforms (M1 and M23) of AQP4 have been identified with different translation initiation sites (12, 18). The shorter M23-AQP4 is the protein responsible for the formation of paracrystalline orthogonal arrays of particles (OAPs) (33) that have been seen by freeze-fracture electron microscopy (FFEM) for more than 30 years (10, 17, 25). The longer M1-AQP4 isoform was shown to oppose the aggregation of the shorter M23-AQP4 because OAPs were significantly smaller in size when these splice variants of AQP4 were coexpressed (7, 27). The relative abundance of the longer M1-AQP4 is tissue specific, suggesting a biological role to inhibit AQP4 assembly into OAPs. It has been speculated that the organization of AQP4 into OAPs might enhance AQP4 water permeability (34) and more recently it was shown that, in LLC-PK1 cells, M1 exhibits significantly lower single-channel water permeability than overexpressed M23-AQP4, which forms typical OAPs (27). However, functional expression of these isoforms in Xenopus laevis oocytes (22) did not suggest a difference in single-channel osmotic water permeability, which may relate to experimental and methodological variation, possibly affecting the paracrystalline organization of AQP4. Interestingly, the organization of AQP4 is perturbed by vasopressin in Brattleboro kidney (27), suggesting that AQP4 membrane organization (and potentially, therefore, function) in tissues where AQP4 is expressed may be under the control of resident hormone receptors. Furthermore, in a rabbit model of ocular hypertension, antagonist studies showed the presence of V2R in the eye (15), which affected intraocular pressure (IOP). IOP is associated with aqueous humor secretion (14) and a major site for this secretion is the ciliary epithelium where AQP4 is expressed (8, 21, 24).

In this study, we investigated whether [lys8]-vasopressin and forskolin can modulate AQP4 OAP organization utilizing stably transfected LLC-PK1 cells expressing wild-type M1 and/or M23 AQP4 isoforms and AQP4 that had undergone site-directed mutagenesis at serine residues that represent putative phosphorylation sites (27). We found differential and time-dependent changes in expression of M1 and M23 splice variants after vasopressin treatment. Coexpression of M1 with M23, or mutation of serine-111, modified the pattern of induced expression. Finally, vasopressin delivery via osmotic minipumps modulated M1 and M23 AQP4 expression in the kidney and also in the eye in Brattleboro rats in vivo.

MATERIALS AND METHODS

AQP4 constructs and transfection into LLC-PK1 cells. The M1 and M23 forms of rat AQP4 containing cassettes were subcloned into 5’-HindIII and 3’-EcoRI sites of pcDNA3.hgyro and pcDNA3.G418 vectors, respectively. Mutations of serine-111 to glycine-111 and glutamic acid-111 to generate M1-AQP4 and M23-AQP4 mutants were performed using the QuickChange site-directed mutagenesis kit.
(Stratagene, La Jolla, CA). Fidelity of the constructs was confirmed by sequence analysis. LLC-PK₁ cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. The procedures to generate stable cell lines included lipofectamine-mediated transfection, selecting in medium containing hygromycin, respectively, geneticin (G418), subcloning, and characterization (27). The generation of cell lines coexpressing M1 and M23 isoforms was accomplished by somatic cell hybridization of M1- and M23-AQP4 cells according to Fig. 1. Vasopressin modulation of orthogonal arrays of particles (OAPs) in wild-type (wt) and S111-mutant aquaporin-4 (AQP4) molecules. LLC-PK₁ cells expressing the M23-AQP4 splice variant exhibit globular shaped patches of OAPs (A; bar = 100 nm), encircled in red. Chronic (3-day) exposure to [lys⁸]-vasopressin and forskolin (LVP/Fk) increased number of OAPs (B; bar = 100 nm). OAPs at higher magnification are drawn floating using lasso-tool-selected (Photoshop) patches enlarged 4-fold relative to the underlying micrograph. LLC-PK₁ cells expressing the M1-AQP4 (wt) do not form OAPs (C; bar = 100 nm) or after LVP/Fk addition (D; bar = 100 nm). The floating rectangle in C shows 2 higher-order aggregates in rectangular arrangement of 4 smaller units, described as incipient arrays (7) of M1-AQP4 molecules and were sporadically detected in untreated cells. M23-S111G mutants (E, F) and M23-S111E (G, H) exhibited highly ordered OAPs of rectangular appearance (lasso-tool-selected floating patches at 4-fold magnification relative to the underlying micrograph), independent of the chronic exposure to LVP/Fk.

Fig. 1. Vasopressin modulation of orthogonal arrays of particles (OAPs) in wild-type (wt) and S111-mutant aquaporin-4 (AQP4) molecules. LLC-PK₁ cells expressing the M23-AQP4 splice variant exhibit globular shaped patches of OAPs (A; bar = 100 nm), encircled in red. Chronic (3-day) exposure to [lys⁸]-vasopressin and forskolin (LVP/Fk) increased number of OAPs (B; bar = 100 nm). OAPs at higher magnification are drawn floating using lasso-tool-selected (Photoshop) patches enlarged 4-fold relative to the underlying micrograph. LLC-PK₁ cells expressing the M1-AQP4 (wt) do not form OAPs (C; bar = 100 nm) or after LVP/Fk addition (D; bar = 100 nm). The floating rectangle in C shows 2 higher-order aggregates in rectangular arrangement of 4 smaller units, described as incipient arrays (7) of M1-AQP4 molecules and were sporadically detected in untreated cells. M23-S111G mutants (E, F) and M23-S111E (G, H) exhibited highly ordered OAPs of rectangular appearance (lasso-tool-selected floating patches at 4-fold magnification relative to the underlying micrograph), independent of the chronic exposure to LVP/Fk.
MacDougall and Matrisian (19) and with hygromycin and geneticin to select for double-resistant colonies (27).

Antibodies and controls. Antibodies against a rat COOH-terminal AQP4 peptide (last 15 amino acids) coupled to keyhole limpet hemocyanin were raised in rabbits. The specificity of this antibody has been reported previously (31). Whole serum was affinity-purified against the immunizing peptide using a Pierce affinity purification column kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Control procedures for immunocytochemistry included incubation of the tissues with preimmune serum and preabsorption of the antibody with the immunizing peptide. For immunoblot analysis of LLC-PK1 cell lines, a commercial polyclonal COOH-terminal anti-AQP4 antibody (Alpha Diagnostics, San Antonio, TX) was used, which was raised against a 17-amino acid sequence, partially overlapping the AQP4 peptide that we used to generate our antibodies.

Vasopressin treatment of LLC-PK1 cells. Cells were grown to 70–80% confluence in 100-mm-diameter dishes, in DMEM, supplemented with 10% fetal calf serum (GIBCO), at 37°C in an atmosphere of 5% CO2. Fresh medium containing 10 nM [lys8]-vasopressin and 10% fetal calf serum (GIBCO) was added, and cells were incubated for another 24 or 72 h at 37°C. Forskolin was added to maximize cAMP production in these cells. In some experiments, cells were incubated for 24 h in DMEM containing 10 μg/ml (in ethanol) cycloheximide, 100 nM (10–7 M) chloroquine, or 6 μM lactacystin. Control non-treated cells were incubated at 37°C during the same periods of time as the treated cells.

Protein extraction. LLC-PK1 cells were harvested with Ca2+- and Mg2+-free PBS containing 5 mM EDTA and centrifuged for 10 min at 1,000 rpm at 4°C. Cell pellets were resuspended in 500 μl of lysis buffer containing (in mM) 0.5 IGEPAL, 10 Tris, 1 EDTA, 1 EGTA, 150 NaCl, 0.2 Na-orthovanadate, and 1% Triton X-100 containing complete protease inhibitors cocktail (Roche) and centrifuged for 15 min at 10,000 rpm.

Immunoblot analysis. SDS-PAGE was performed using an Invitrogen/Xcell Mini Cell electrophoresis system using the Laemmli-type biphasic buffer system (16) to cast our own 10% acrylamide/Tris-glycine (pH 8.8)/0.1% SDS (see Figs. 2, 4, 5, 12) or 12% acrylamide/4M-urea/Tris-glycine (pH 8.8)/0.1% SDS (see Fig. 6) gels for destacking proteins. This system allows better resolution of the M1 and M23 isoforms of AQP4. Samples were prepared at room temperature in SDS-PAGE application buffer containing 0.5% β-mercaptoethanol. For Western blotting, proteins were transferred to an Immobilon PVDF membrane by electrophoresis at 5–7 V for 18 h in 1 mM Na-carbonate, 4 mM Na-bicarbonate, and 0.01% SDS (pH 9.9). The membrane was blocked by a 1-h incubation in PBS, 0.05% Tween, containing 5% nonfat milk. The membrane was then incubated for 2 h with a 1:200 dilution of anti-AQP4 antibody, washed with blocking solution, and incubated for 1 h with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody in the presence of the blocking solution. The blots were washed with PBS and assayed for peroxidase activity, using the Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear, Boston, MA).

Fig. 2. Immunoblots of total LLC-PK1 cell homogenates resolved on SDS gels. Antibody staining against AQP4 (top blots) and β-actin (bottom blots) are shown in A, containing 6 samples at 3 different protein concentrations (25, 5, 1 μg). The first 2 samples represent M23 wt, the second 2 S111E M23 mutants, and the third 2 represent S111G M23 mutants treated without or with LVP/Fk for 3 days. B: cell homogenates at 25, 5, 1 μg protein of M1-AQP4-expressing cells, treated with LVP/Fk for 0, 1, and 3 days. C: homogenates of cells expressing M1 S111G and S111E mutants treated for 0, 1, and 3 days with LVP/Fk. Note that only wt M1 and M23-AQP4 expression levels are modulated by vasopressin. D summarizes quantitatively vasopressin-induced differential M1 and M23 expression. The data were normalized to untreated control samples and the bar graph of relative LVP/Fk-induced AQP4 expression at 1 and 3 days LVP/Fk treatment depicts the means and SE. Student’s t-tests indicate significant differences (P < 0.001, n = 3) between 1 and 3 days of treatment.
Experimental animals. Animal experiments were approved by the Institutional Committee on Research Animal Care, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tissues from Brattleboro rats, anesthetized with pentobarbital sodium (65 mg/kg ip), were fixed by intravascular perfusion with parafomaldehyde lysine periodate (PLP) fixative as previously described (1). After fixation, tissues were washed and stored in PBS (0.02% Na-azide, 10 mM sodium phosphate buffer, 0.9% NaCl, pH 7.4). For immunoblot analysis, membrane fractions, obtained by differential centrifugation of fresh tissue homogenates, were snap-frozen and stored at −80°C.

Chronic vasopressin treatment of Brattleboro rats. Adult male, vasopressin-deficient Brattleboro homozygous rats weighing 300–360 g were used. The Brattleboro rats were divided into two groups (6 animals/group). One group of animals was not treated (control) and the other received the vasopressin analog 1-desamino-8-D-arginine vasopressin (dDAVP) subcutaneously at a rate of 5 μl/h (1.2 μg/day) via osmotic minipumps (27). All animals had free access to food and water for the duration of the studies.

RESULTS

Characterization of rat AQP4 splice variants (M1 and M23) and S111G and S111E mutants in stably transfected LLC-PK1 cell lines. M23-AQP4-expressing cells exhibited typical orthogonal arrays as seen by FFEM similar to those previously associated with AQP4 expression in membranes (Fig. 1A). Treatment with [lys8]-vasopressin plus forskolin (LVP/Fk) for 3 days increased the number of OAPs in LLC-PK1 cells stably transfected with M23-AQP4 (Fig. 1B). In contrast, freeze-fracture of M1-AQP4-expressing LLC-PK1 cells showed many intramembranous particles (IMPs) of uniform size (Fig. 1C) that were similar to control, untransfected LLC-PK1 cells (not shown). However, inspection of membrane replicas at higher magnification did reveal some incipient, small arrays of IMPs (Fig. 1C, floating inset) that have been described earlier in Chinese hamster ovary cells expressing M1-AQP4 (7). After

Fig. 3. Vasopressin modulation of M1 and M23 interaction. Somatic hybridization of M1- and M23-expressing cells resulted in hybrid cell lines, clone #9 (A, B, C) and clone #14 (D, E, F), expressing M1 and M23 in 1:1 ratios. A (bar = 100 nm) and D (bar = 25 nm) show small-sized OAPs that were increased significantly in number (B: bar = 100 nm, E: bar = 25 nm) after 1-day exposure to LVP/Fk. A 3-day LVP/Fk treatment did not result in significant increase of OAP number (C: bar = 100 nm, F: bar = 25 nm).
chronic exposure (3 days) to LVP/Fk (Fig. 1D) such incipient arrays on the membrane were not detected (not shown). LLC-PK₁ cells stably expressing S111G- and S111E-AQP4 M23 mutants showed quadrangular lattices of very orderly OAPs of sizes that were up to 2.5-fold larger (Fig. 1, E–H) than the wild-type OAPs (Fig. 1A). Immunoblotting of total cell extracts (Fig. 2) revealed a more than fourfold increase in M23-AQP4 expression after a 3-day exposure to LVP/Fk (Fig. 2A, first 2 sets of experiments), consistent with the increased occurrence of oblong-shaped OAPs (Fig. 1A). However, expression levels of M23-AQP4-S111G and -S111E mutants were not affected by chronic LVP/Fk exposure (Fig. 2A, last 4 sets of experiments). In contrast to the M23 isoform, M1-AQP4 expression (Fig. 2B) was maximal after 1 day and decreased to control levels after a 3-day LVP/Fk exposure. Similar to mutant M23-AQP4, apparent expression levels of S111G and S111E M1-mutants were not affected by LVP/Fk (Fig. 2C). Quantitative immunoblot densitometry (Fig. 2D) summarizes these two distinct patterns of induced expression of M1- and M23-AQP4.

M1/M23 coexpression in hybrid cell lines. Somatic hybridization of LLC-PK₁ cells expressing M1 and M23 isoforms was carried out to investigate the apparent effect of M1 to oppose or inhibit OAP formation by M23-AQP4. Following cloning of four cell lines (#5, #9, #11, #14), freeze-fracture revealed the occurrence of small patches of OAPs (#9; Fig. 3A, #14; Fig. 3D). These OAPs were significantly increased in number after a 1-day (Fig. 3, B and E) but not after a 3-day LVP/Fk treatment (Fig. 3, C and F). The increase in number of OAPs after 1 day was reminiscent of the noticeable increase of OAPs reported in Brattleboro rats following dDAVP administration for 1 day (27). Immunoblot analysis of whole cell homogenates of the clones showed higher M1 levels of expression in clone #5 and #11 and about equal levels of M1 and M23 in clone #9 and #14 (Fig. 4, A–D). Some of these hybrid cell lines showed that maximum levels of both M1 and M23 variants were detected after 1-day LVP/Fk treatment, when normalized to β-actin content. While vasopressin-modulated M1 expression in hybrid cell lines (Fig. 4, E–F) was not different from that in M1-expressing cells (Fig. 2B), the vasopressin-modulated expression of M23 in hybrid cells (Fig. 4, E–F) followed the behavior of M1, i.e., it was increased after 1 day but not after 3 days. This pattern was different from the modulated expression of M23 in cells expressing the M23 variant alone (Fig. 2A). Quantitative densitometry of blots is summarized in Fig. 4G and shows the differential vasopressin-induced expression response when M1 and M23 are expressed alone, compared with the similar isoform response when M1 and M23 variants were expressed together.

One possible mechanism for increased expression of AQP4 is decreased degradation upon internalization. However, the increase in AQP4 splice variant protein by vasopressin was not sensitive to chloroquine (Fig. 5), a lysosomal inhibitor. M1-induced expression levels in M1 or M1/M23 cells and M23-induced expression levels in M23 or M1/M23 cells by 1-day LVP/Fk treatment plus chloroquine were almost identical to...
the induced levels when chloroquine was absent. A similar result was obtained with lactacystin, a proteasome inhibitor (not shown). However, treatment of cells with cycloheximide to block protein synthesis prevented vasopressin-induced increases in AQP4 expression after 1 day. Data extending to 3 days LVP/Fk stimulation in the presence of an inhibitor were excluded from this study because of the toxicity of cycloheximide after such prolonged treatment.

We next examined the effect of in vivo administration of vasopressin on AQP4 expression to Brattleboro rats in vivo. Immunoblot analysis of membrane preparations of renal tissues from these rats showed a significant decrease in M1 expression and an increase in M23 expression following chronic treatment (8-day) with vasopressin (Fig. 6A, left). Densitometric analysis of immunoblots derived from SDS gels containing 4 M urea indicated an M1/M23 ratio of 0.63 before vasopressin and 0.76 after vasopressin stimulation. In the outer medulla, a more than 10-fold increase of the 31-kDa band (M23) was observed, while M1 was not detectable in this region of the kidney. Urea SDS gels also revealed significant expression of the AQP4 M1 isoform in brain and kidney tissues of Sprague-Dawley rats and B6 mice. In addition, another putative AQP4 isoform, seen as a faint band at 41 kDa (20, 22), could be detected. While immunoblots of Brattleboro rat inner medulla showed abundant presence of AQP4 before and after treatment with dDAVP, immunocytochemistry revealed, however, absence of AQP4 staining in the inner medulla of vasopressin-treated Brattleboro rats (Fig. 6B). This discrepancy suggests a vasopressin-induced epitope shielding of AQP4 in the inner medulla. Shielding of the COOH-terminal AQP4 epitope by vasopressin may also be applicable to the ciliary epithelium of the eye (Fig. 6C), which also showed a significant decrease in AQP4 staining following vasopressin treatment. Interestingly, this could suggest altered aqueous humor dynamics affecting aqueous inflow and IOP.

In follow-up experiments, 8-day vasopressin-infused rats were subjected to “vasopressin washout” by removal of the implanted osmotic pumps. After 1 and 4 days, the urinary osmolality was reduced to 40 and 100 mosmol/kgH2O, respectively, and AQP4 immunostain reappeared on principal cells of the inner medulla, indistinguishable (not shown) from untreated Brattleboro rats. In Long Evans rats, AQP4 staining in inner medulla was robust (not shown).

DISCUSSION

We showed that M1 and M23 isoforms of AQP4 display distinct vasopressin-induced expression levels when expressed alone in LLC-PK1 cell lines. The selective increase in M1-AQP4 occurrence at early stages and decrease at later stages of the vasopressin response, compared with a gradual and sustained increase of M23-AQP4, may be related to changes in AQP4 assembly and/or expression in Brattleboro rats that were subjected to chronic dDAVP treatment (27). The M1:M23 stoichiometry we propose is based on the observation that the clearly different pattern of vasopressin-induced M1 and M23 expression in different cell lines was abolished when M1 and M23 were coexpressed in equal amounts. We postulate that the M1/M23 higher-order unit (hetero-octomer, 1 M1-tetramer plus 1 M23-tetramer) forms the basis of a complex OAP regulation in vivo. This model excludes the possibility that
OAPs can consist of M1-M23 heterodimers; OAPs are noninterconvertible assemblies of M23-AQP4 molecules as was shown by quantum dot single particle tracking (4).

Mechanism of AQP4 regulation at the onset and sustained presence of vasopressin. In an in vivo environment where most of AQP4 is expressed as the M23 splice variant, the formation of M1/M23 higher-order units upon vasopressin-induced up-regulation of M1 would rapidly increase the number of M1/M23-AQP4 higher-order units at the expense of M23/M23-AQP4 higher-order units existing in orthogonal arrays. This mechanism may result in an increase in small-sized orthogonal arrays such as were seen in Brattleboro rats after 1-day vasopressin treatment (27). Because the increase of AQP4 orthogonal arrays in hybrid cell lines by the vasopressin response was transient, it could mean that M1/M23 and/or M1/M1 higher-order units that are not arranged into large OAPs are more prone to endocytosis and subsequent routing to compartments involved in protein degradation. Assuming that M1/M23 higher-order units disappear on chronic administration of vasopressin to Brattleboro rats, the remaining M23-AQP4 molecules, still in OAPs, will be subsequently complexed with newly recruited M23-AQP4 as the result of a sustained response to vasopressin.

The result of this chronic response would be to selectively increase the M23-AQP4 content of membranes. Immunoblot analysis in this study and freeze-fracture analysis in our previous study (27) support this selective increase of M23-AQP4 protein expression with an increase in OAP size after an 8-day administration of vasopressin.

Exogenous protein expression and endogenous pathways. There are a number of considerations when comparing overexpressed AQP4 in a cell line to endogenously expressed AQP4 in Brattleboro rats. The introduction of exogenous AQP4 cDNA with its bacterial derived promoter in LLC-PK1 cells led to the production of AQP4 protein, a process that was differentially regulated by the vasopressin-signaling pathway for the M1 and M23 isoforms. However, because the exogenous cDNA constructs do not contain introns, native promoters, and putative transcriptional elements of the aquaporin 4 gene, their transcription would be predicted to yield AQP4 RNAs specific to the M1- and M23-cDNAs and that are not controlled by the addition of vasopressin. However, because the final levels of M1 and M23 are regulated differently, it implied a posttranslational mechanism to explain our findings. This could occur by selective translation of the RNAs, consistent
with our data showing that protein synthesis is necessary for the effect to be detected, or by differential degradation, although we were unable to show any effect of degradation pathway inhibitors in this system. Finally, mRNA stability could also be involved. Another factor is the notion that principal cells of the collecting duct express significantly more M23-AQP4 than M1-AQP4, contrary to the stable cell lines. However, if one M1 molecule interacts strongly with one M23 molecule in vivo, then by concept there will be a cohort of M23-AQP4 molecules, assembled into OAPs that can be regarded as being distinct from the M1/M23 hetero higher-order unit. Vasopressin elevates cAMP, induces higher levels of protein expression, activates PKA, and induces phosphorylation of proteins, which could in some way result in the increased levels of foreign AQP4 expression that we observe here, and which is sensitive to cycloheximide and an intact serine-111 residue. Serine-111 is part of a putative phosphorylation motif and an estimation of relative AQP4 expression between wild-type and mutant AQP4 cell lines suggested that S111E-AQP4 cell lines had a higher content of AQP4 than S111A cell lines (27). This would support the notion that S111 is subject to phosphorylation/dephosphorylation events under control of vasopressin/PKA action, but the mechanism by which this occurs remains to be determined.

The differential vasopressin-induced increase in steady-state expression of the AQP4 splice variants may be explained by different turnover rates as the result of a difference in the phosphorylation rate of the M1- and M23-AQP4 isoforms. For example, phosphorylation of M23-AQP4 might lag behind that of M1-AQP4 due to selective shielding of S111, which would prevent the creation of an endocytotic block that involves a reduced capacity of phosphorylated AQP4, similar to phosphorylated AQP2-S256, to interact with the endocytotic machinery of the overexpressed LLC-PK1 cell (2). Our study suggests that the rate-limiting step of foreign protein turnover is upstream of the protein degradation pathway, and downstream of the transcription and translation pathway. We postulate that the turnover of AQP4 splice variant M23 lags behind that of M1 because it assembles into OAPs, which by spatial considerations may not be easily endocytosed from the cell plasma membrane, unless these OAPs are disrupted. In a previous study, we showed that AQP4 in the form of OAPs is long-lived in the basolateral membranes of gastric parietal cells (11). Also, a larger cell plasma membrane surface was observed both in our LLC-PK1 cells expressing the AQP4 splice variant M23 and in astrocytes, compared with control or M1-LLC-PK1 cells, respectively, or AQP4-RNAi-treated astrocytes (23, 27). Taken together, our in vitro data suggest that AQP4 splice variants and vasopressin have roles in the regulation of the in-membrane OAP assembly and, by extension, water permeability.

AQP4 conformational changes induced by vasopressin. Of note is the apparent disappearance, observed by immunocytochemistry, of anti-AQP4 antibody staining in the inner medulla after vasopressin treatment, in contrast to immunoblot analysis showing a sustained signal both before and after vasopressin stimulation. This most likely represents an epitope-shielding effect potentially due to a conformational change in the AQP4 COOH terminus. This conclusion is corroborated by freeze-fracture analysis (27) where it was shown that OAP structure was significantly affected by the chronic presence of vasopressin. Reduced AQP4 immunocytochemical reactivity in ciliary epithelium of the Brattleboro rat eye in response to chronic exposure of dDAVP, as shown in this study, and studies on urea transporter expression by vasopressin in Brattleboro rats with a COOH-terminal anti-urea transporter antibody (26), may be other examples of epitope shielding. Conversely, immunoblot studies in chronic vasopressin-treated Brattleboro rats on α-EnaC expression (5) showed no increase vs. increases of β- and γ-EnaC subunits in whole kidney homogenates. Also, immunoblot analyses using COOH-terminal antibodies against AQP1–4 have been used to map aquaporins in Brattleboro rats, showing that AQP1 and AQP4 staining is unaltered after vasopressin (13, 28). These data are in agreement with our immunoblot analysis of AQP4 in outer and inner medulla, showing no change of total AQP4 (M1 + M23) in inner medulla. However, the 10-fold increase of AQP4 content in the outer medulla, determined both by immunocytochemistry and immunoblots that we report here, is novel. Support for altered AQP4 immunoreactivity (increased) was also found in rat brain in response to systemic hyponatremia (29), suggesting that AQP4 conformation is highly sensitive to the physiological state of the animal.

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