Membrane organization and function of M1 and M23 isoforms of aquaporin-4 in epithelial cells

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Silberstein, Claudia, Richard Bouley, Yan Huang, Pingke Fang, Nuria Pastor-Soler, Dennis Brown, and Alfred N. Van Hoek. Membrane organization and function of M1 and M23 isoforms of aquaporin-4 in epithelial cells. Am J Physiol Renal Physiol 287:F501–F511, 2004. First published May 18, 2004; 10.1152/ajprenal.00439.2003.—Aquaporin-4 (AQP4) water channels exist as heterotetramers of M1 and M23 splice variants and appear to be present in orthogonal arrays of intramembranous particles (OAPs) visualized by freeze-fracture microscopy. We report that AQP4 forms OAPs in rat gastric parietal cells but not in parietal cells from the mouse or kangaroo rat. Furthermore, the organization of principal cell OAPs in Brattleboro rat kidney is perturbed by vasopressin (arginine vasopressin). Membranes of LLC-PK1 cells expressing M23-AQP4 showed large, abundant OAPs, but none were detectable in cells expressing M1-AQP4. Measurements of osmotic swelling of transfected LLC-PK1 cells using videomicroscopy, gave osmotic water permeability coefficient ($P_f$) values (in cm/s) of 0.018 (M1-AQP4), 0.019 (M23-AQP4), and 0.003 (control). Quantitative immunoblot and immunofluorescence showed an eightfold greater expression of M1 over M23-AQP4 in the cell lines, suggesting that single-channel $p_f$ ($cm^2/s$) is much greater for the M23 variant. Somatic fusion of M1- and M23-AQP4 cells ($P_f = 0.028 cm/s$) yielded OAPs that were fewer and smaller than in M23 cells alone, and M1-to-M23 expression ratios (~1:4) normalized to AQP4 in M1 or M23 cells indicated a reduced single-channel $p_f$ for the M23 variant. Expression of an M23-AQP4-Ser118E mutant produced ~1.5-fold greater single-channel $p_f$ and OAPs that were up to 2.5-fold larger than wild-type M23-AQP4 OAPs, suggesting that a putative PKA phosphorylation site Ser118 is involved in OAP formation. We conclude that the higher-order organization of AQP4 in OAPs increases single-channel osmotic water permeability by one order of magnitude and that differential cellular expression levels of the two isoforms could regulate this organization.

water transport; freeze-fracture; LLC-PK1 cells; orthogonal arrays; intramembranous particles

THE MAMMALIAN FAMILY OF AQUAPORIN water channels consists of 12 known members, each with a specific tissue distribution and membrane localization pattern. However, the role of aquaporin-4 (AQP4) in water transport physiology is not well understood. AQP4 was the first aquaporin to be observed and identified in biological membranes, because when examined by freeze-fracture electron microscopy, it forms characteristic arrays of intramembranous particles (IMPs) in the form of checkerboard aggregates or orthogonal arrays of intramembranous particles (OAPs). These OAPs were described in various cell membranes long before a role in water permeability was suspected (3, 18, 27, 31, 32). The relationship of OAPs to water channels was first suggested by earlier studies showing that membranes that contained OAPs, including astrocytes, gastric parietal cells, and collecting duct principal cells were immunostained by an antibody raised against the whole AQP1 (then called CHIP28) protein (37). A different antibody raised against skeletal muscle OAP-containing membranes also recognized an ~30-kDa protein in these membranes (15, 41). In this way, a protein initially called basolateral intrinsic membrane protein (BLIP) was identified in the basolateral membranes of kidney collecting duct principal cells (37). BLIP was recognized to be AQP4 after the cloning and identification of this novel, mercurial-insensitive water channel (8, 14, 19). OAPs also closely resemble frog skin vasopressin- and isoproterenol-induced IMP aggregates related to transepidermal water flow (4). The observation that OAPs are found in plasma membranes of CHO cells transfected with AQP4 cDNA (44) and that OAPs are not found in AQP4 knockout mice (40) provided compelling evidence that these arrays are indeed composed of AQP4 proteins. This was subsequently confirmed directly by elegant freeze-fracture/immunolabeling studies on astroglial OAPs (33).

AQP4 (−/−) mouse knockout models suggested that AQP4 plays a role in hearing (21, 26). However, AQP4 deletion did not reveal any critical role for this channel in urinary concentration, acidification of the gastric lumen, or skeletal muscle function (6, 42, 45). A negative role in this context was that deletion of AQP4 reduced brain edema after acute water intoxication and ischemic stroke (25). However, in vivo measurements of transepithelial $P_f$ in microdissected inner medullary collecting ducts (IMCDs) to measure AQP4 function have been carried out after 18–48 h of water deprivation in the presence of arginine vasopressin to make basolateral $P_f$ rate limiting (6). Transepithelial $P_f$ was reduced fourfold in AQP4-deletion mutants, indicating that AQP4 can play a role in transepithelial volume flow under some circumstances.

Studies of AQP4 localization in the rat, mouse, and kangaroo rat showed a species-dependent distribution of AQP4 protein and OAPs in the kidney. In the rat, AQP4 occurs only in basolateral membranes of principal cells; whereas in mouse, AQP4 protein is also present basolaterally in proximal tubules of the S3 segment (38). However, whereas OAPs are abundant in principal cells of both species, only a few, very small OAPs were detectable in mouse proximal tubule cells. Furthermore, AQP4 protein was not detectable in the kidney of kangaroo rats. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
but was present in stomach and brain of these animals. Kangaroo rats, which exhibit a kidney-specific AQP4 “knockout” phenotype, concentrate their urine up to 6,000 mosmol/kgH2O, underlining the absence of a critical role for AQP4 in urinary concentration. At the transcriptional level, however, AQP4 mRNAs were found in collecting duct principal and proximal tubule cells of both the mouse and kangaroo rat (16).

In addition to cell- and species-specific expression of AQP4 and OAPs, some reports have shown that the number and appearance of OAPs in cell membranes can be regulated under various conditions. For example, the number of OAPs in principal cells of rat kidney is increased by acute dehydration of the animal (28). OAP number is also increased in rat stomach exposed to pentagastrin, which increases acid secretion (2). Disruption of OAP structure in astrocyte membranes is an early event in acute global ischemia (35). These data suggest that OAP formation and/or AQP4 expression is regulated physiologically.

However, only limited knowledge exists with respect to the role of M1-AQP4 and M23-AQP4 variants that are coexpressed in several cells (19). M1-AQP4 and M23-AQP4 are splice variants (22), and in native tissues, they form heterotetramers of individual 34- and 32-kDa functional units (29). Heterotetramers appear in freeze-fracture electron microscopy as IMPs, and M23-AQP4 is the abundant polypeptide found in native tissues (16, 29, 38). The relative role of these variants in OAP formation is unknown, although M23-AQP4 alone is clearly able to form OAPs in the plasma membranes of transfected cells (39, 44).

The apparent complexity of AQP4 expression with respect to species-dependent tissue distribution and morphology, the occurrence of spliced forms, and the lack of detailed functional data prompted us to evaluate the role of the M1 and M23 variants in OAP formation and water channel activity. We found AQP4 to occur in three distinct morphological states depending on the species and organ system examined, and on physiological conditions. Stably transfected LLC-PK1 cells expressing M1- and M23-AQP4 proteins demonstrated that M23-AQP4 is the OAP-forming water channel, and that M23-AQP4 when organized in OAPs has a significantly higher single-channel water permeability coefficient than its counterpart M1-AQP4, which does not form OAPs.

MATERIALS AND METHODS

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 mouse (New Zealand White) and kangaroo rat (Dipodomys merriami) were removed after euthanasia with isoflurane, cut into 2- to 3-mm slices, and immersion fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 8 h (17). Tissues from Sprague-Dawley, Long-Evans, and Brattleboro rats anesthetized with pentobarbital sodium (65 mg/kg ip) were fixed by intravascular perfusion with paraformaldehyde/lysine periodate 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 freeze-fracture electron microscopy of tissues from Brattleboro and Long-Evans rats, intravascular perfusion was carried out with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.4). After a 5-min perfusion in situ, tissues were removed and cut into 2- to 3-mm slices that were fixed for a further 6 h with glutaraldehyde. 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 four groups (6 animals/group: 3 for freeze-fracture electron microscopy and 3 for immunocytochemistry). One group of animals was not treated (control), and the other three groups 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, as previously described (34). This dose has been shown to produce plasma vasopressin levels comparable to those achieved in normal rats during water restriction (10). Animals from two of these dDAVP groups were fixed after either 1 day (acute) or 8 days (chronic) dDAVP exposure. The final dDAVP group was treated for 8 days, and then the minipumps were removed (dDAVP washout). Two of these rats were fixed 1 day later, and 4 rats were fixed 4 days later. All Brattleboro rats had free access to food and water for the duration of the studies. A 5500 Wescor vapor pressure osmometer (Wescor, Logan, UT) was used to measure urine osmolality. Urine samples were collected by “clean catch” before and after the implantation of the minipumps, and at various times during the 8- to 9-day treatment period.

AQP4 constructs and transfection into LLC-PK1 cells. The M1 and M23 forms of AQP4-containing cassettes were subcloned into 5’- HindIII and 3’-EcoRI sites of pcDNA3hygro and pcDNA3G418 vectors, respectively. Mutations of Ser111 to Gly111 and glutamic acid111 to generate M1-AQP4 and M23-AQP4 mutants were performed by using a 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.

To obtain stable AQP4-expressing cell lines (LLC-AQP4), LLC-PK1 cells were plated at a density of 150,000/60-mm dish, 20 h before transfection. For transfection, lipofectamine (15 μl) with 4 μg of plasmid DNA was added to the cells, incubated at 37°C for 4 h, and washed once with serum-free DMEM. After 14–20 days of selection in medium containing 400 μg/ml hygromycin or 1 mg/ml of Geneticin (G418), respectively, resistant colonies were isolated by dilution series and transferred to separate culture dishes for expansion. Several clones were isolated and characterized for AQP4 expression using the anti-AQP4 COOH-terminal antibody described below (which does not distinguish between the M1 and M23 isoforms).

Somatic cell hybrids of M1- and M23-AQP4 cells were obtained according to MacDougall and Matrisian (23). Cells were seeded together at a density of 5 × 105 each in a 60-mm tissue culture plate containing growth medium and allowed to attach overnight. Cells were exposed for 1 min to 50% polyethylene glycol 2,000/10% DMSO in PBS (Sigma), washed several times with PBS, and allowed to recover overnight in DMEM. Cells were harvested and plated in DMEM supplemented with hygromycin and Geneticin to select for double-resistant colonies.

Antibodies and controls. Antibody against a rat COOH-terminal AQP4 peptide (last 15 amino acids) coupled to keyhole limpet hemocyanin was raised in rabbits. The specificity of this antibody has been reported previously (16). Whole serum was affinity purified against the immunizing peptide using an 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 a commercial 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 we utilized to generate our in-house anti-AQP4 antibodies.

Immunocytochemistry. LLC-PK1 cells were fixed in 4% paraformaldehyde for 20 min, washed in PBS, and incubated overnight with...
primary antibodies. After washing, secondary antibodies Cy3-conjugated goat anti-rabbit IgG (2 μg/ml final concentration; Jackson ImmunoResearch, West Grove, PA) were then applied for 1 h at room temperature. Coverslips were mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA) diluted 1:1 with Tris-HCl, pH 8.9. Cells were examined by using a Nikon 800 Eclipse photomicroscope, and images were captured by using a Hamamatsu Orca charge-coupled device camera. Digital images were stored and analyzed by using IP Lab Spectrum imaging software (Signal Analytics, Fairfax, VA) and Adobe Photoshop (16, 17).

Freeze-fracture electron microscopy. LLC-PK₁ cells were fixed by immersion for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissues were fixed as described above. After cryoprotection for at least 1 h in 30% glycerol, cells were scraped from the culture dish, and clumps of cells were placed on a copper freeze-fracture support and frozen in Freon 22 cooled by liquid nitrogen. Small pieces of tissues were similarly cryoprotected and frozen on copper supports. Freeze-fracture replicas from tissues or cells were produced as previously described (38, 39). After removal from the freeze-fracture device, the replicas were cleaned by immersion for 2 h in concentrated sodium hypochlorite bleach. Replicas from tissues were treated with chloroform/methanol mixtures for further extraction of unwanted material. Replicas were washed three times for 5 min each with distilled water, picked up on copper electron microscopy grids, and examined with a Philips CM10 electron microscope. Areas of plasma membranes from 20 control cells and 20 AQP4-transfected cells and areas from tissues from 3 to 6 animals were photographed at a final magnification of ×28,000. The area occupied by OAPs was measured and expressed as a percentage of the total membrane surface area.

Functional measurements. LLC-PK₁ cells and stably transfected LLC-PK₁ cells expressing M1-AQP4 and M23-AQP4 water channels were seeded on coverslips and after 24 h were mounted in a perfusion chamber designed to accommodate coverslips. The chamber includes holders for suction tubing. The perfusion rate (0.3–0.5 ml/s) was controlled gravitationally, and a switching system was used to rapidly change solutions of different composition. Perfusion rates were fast enough to reduce mixing/exchange times to <1 s and slow enough to not deform cells during mixing and liquid exchange. Video-enhanced

Fig. 1. Comparative immunolocalization of aquaporin-4 (AQP4) in gastric glands. Indirect immunocytochemistry was carried out with anti-AQP4 primary and Cy3-conjugated secondary goat anti-rabbit antibodies. Panels indicate basolateral staining of a subset of parietal cells in Sprague-Dawley rat cells (2/3 of the cells are stained; A), Brattleboro rats (most parietal cells are stained; B), New Zealand White mouse (1/3 of parietal cells are stained; C), and kangaroo rat, Dipodomys merriami (most parietal cells are stained; D). Bar = 15 μm.
differential interferential contrast (DIC)-microscopy was carried out with a Nikon inverted microscope (Eclipse TE 300) using short working-distance DIC objectives. Images were obtained through a Hamamatsu digital camera and controller, interfaced through the Mac OS 9.2 operating system and Openlab 3.0 software (Improvision, Boston, MA). Real-time images were obtained every second and stored as QuickTime movies. Each image was retrieved, and the diameter of cell images was measured in pixels and converted to a radius ($r$). Treating the cells as spheres, $P_f$ was determined from initial rate analysis using

$$P_f \approx \frac{1}{r_0^2} V_w c_s \frac{r}{r_0} \Delta t$$

where $r$ is the cell radius (cm), $r_0$ is the initial radius, $t$ is time (s), $P_f$ is the osmotic water permeability coefficient (cm/s), $V_w$ is partial specific volume of water (18 cm$^3$/mol), and $c_s$ is the concentration difference (mosmol/g).

Quantitative immunofluorescence. The relative surface expression of M1- and M23-AQP4 per unit area was obtained from fluorescent-labeled cell membranes using identical immunostaining and image capture parameters. Cell-plating conditions were identical to those used for the functional measurements. A region of interest (ROI) representing the contour of each individual cell membrane examined was outlined utilizing IP Lab software, and the mean pixel intensity within the ROI was taken as a measure of unit density of AQP4 at or near the plasma membrane. This procedure was repeated on batches of cells incubated by using serial antibody dilutions, and traces of pixel intensity vs. antibody dilution were obtained for M1- and M23-expressing cell lines. These traces were used to calculate the relative expression levels of M1- and M23-AQP4 in the LLC-PK1 cells.

Immunoblot analysis. LLC-PK1 cells grown to confluency, were lysed with IGEPAL buffer [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 inhibitor cocktail (Roche)]. The extracts were cleared by centrifugation and aliquots were snap frozen. Also, aliquots of IGEPAL-soluble membrane fractions from native tissue were prepared and frozen. SDS-PAGE was performed by using an Invitrogen/Novex Xcell Mini Cell Electrophoresis System and 10% acrylamide/Tris-glycine/SDS cast gels. Samples were prepared at room temperature in SDS-PAGE application buffer containing 5.0% β-mercaptoethanol. For Western blot analysis, proteins were transferred to an Immobilon PVDF membrane by electrophoresis at 5–7 V for 18 h in 1 mM Na-carbonate, 4 mM NaHCO$_3$, and 0.01% SDS, (pH 9.9). The membrane was blocked by a 1-h incubation in PBS (0.05% Tween) containing 5% (wt/wt) 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).

RESULTS

Species-specific distribution of AQP4 and OAP formation in gastric parietal cells. Immunocytochemistry of gastric tissues of a Sprague-Dawley rat (Fig. 1A), Brattleboro rat (Fig. 1B), mouse (Fig. 1C), and kangaroo rat Dipodomys merriami (Fig. 1D) utilizing anti-AQP4 antibodies showed a species-dependent distribution of AQP4 in parietal cells. Parietal cells were identified by staining with anti-H+/K+-ATPase antibodies (not shown, cf. Refs. 16 and 17). Most parietal cells in Brattleboro rat stomach contained AQP4 (Fig. 1B), whereas only the lower one-third of parietal cells in mouse gastric glands were positive (Fig. 1C). In Sprague-Dawley rats, an intermediate pattern was seen, with about two-thirds of the parietal cells expressing AQP4 (Fig. 1A). Almost all parietal cells in kangaroo rat gastric glands were intensely stained for AQP4 (Fig. 1D).

Immunoblot analysis did not reveal any apparent difference in the relative intensities of the 31- and 34-kDa bands in gastric glands from Sprague-Dawley rat and mouse stomach (not shown).

In freeze-fracture electron microscopy, membranes of Sprague-Dawley rat parietal cells contained orthogonal array particle (OAP) aggregates (Fig. 2A), which consisted of IMPs arranged along two orthogonal axes of symmetry, giving them a checkerboard appearance. OAPs were also abundant in control Brattleboro rats (Fig. 2B, left), but chronic vasopressin treatment (8 days) reduced the number of parietal cell OAPs (Fig. 2B, right). Immunoblot analysis did not indicate detectable changes in M23-to-M1 ratios or reduction after 8-day AVP in parietal cells (not shown). Replicas from a mouse (Fig. 2C) and kangaroo rat (Fig. 2D) did not reveal any obvious OAPs in parietal cells. For comparison, replicas of basolateral membranes of collecting-duct principal cells from Sprague-Dawley rats (Fig. 2A, inset) and mouse (Fig. 2C, inset) contained numerous OAPs.

Chronic dDAVP treatment perturbs the organization of OAPs in Brattleboro rat collecting-duct principal cells. Our preliminary observations suggested that OAPs were especially prominent in AQP4-expressing tissues from homozygous Brattleboro rats that lack vasopressin, implying that this hormone might have some role to play in their appearance or formation. In untreated rats, protoplasmic (P) faces were speckled with OAPs (Fig. 3A), and in exoplasmic (E)-face views numerous OAP imprints were detected (not shown). After 1 day of vasopressin infusion, urine osmolality increased from 100 to 1,000 mosmol/kg H2O and, in some replicas, the number of OAPs appeared to be increased (Fig. 3B), as previously described (28). However, replicas from inner (Fig. 3C) and outer (Fig. 3D) medulla after a more chronic 8-day infusion of vasopressin (urinary osmolality ~2,200 mosmol/kg H2O) showed a considerable reduction in the number of detectable OAPs. The membrane area occupied by these structures fell from 10.7 (control) to 4.5% (dDAVP treated). The control value is similar to that previously reported in basal membranes of principal cells (31). In parallel, numerous irregular and elongated P-face aggregates, occupying 3.2% of the basolateral membrane area, appeared in principal cell mem-
branes (Fig. 3, C and D, white arrows). Inspection of E-face views, however, still revealed many imprints of OAPs, reminiscent of the typical AQP4 pattern (Fig. 3E, black arrows). As shown in Fig. 3F, elongated imprints were also found, suggesting that the “irregular” IMP aggregates in the P face were in fact AQP4 aggregates, but not in the form of typical OAPs. Fortuitous replicas containing both P- and E-face views of basolateral membranes (Fig. 4) confirmed the parallel presence of many irregular IMP clusters (but few if any OAPs) on the P-face and numerous typical OAP-imprints in E-face views. This supports the idea that the irregular IMP aggregates on the P-face represent AQP4 molecules that have a perturbed P-face structural organization after chronic dDAVP exposure. Immunoblot analysis (not shown) indicated insignificant changes in AQP4 content compared with control, asserting the notion of perturbed P-face OAPs.

M23-AQP4 is the OAP-forming variant of AQP4. Stably transfected LLC-PK1 cells expressing M1- and M23-AQP4 water channels were examined by freeze-fracture electron microscopy and by immunofluorescence microscopy. Both M1- and M23-AQP4 were expressed on basolateral plasma membranes of the respective cell lines (Figs. 5, A and B). The nuclear labeling seen in these cells was nonspecific (Fig. 5, C and D), because it was not abolished or diminished when cells were incubated with anti-AQP4 antibodies that had been pre-absorbed with the immunizing AQP4 peptide. Expression levels of M1-AQP4 were seven to eight times higher than M23-AQP4 as quantified by fluorescence intensity measurements of

![Image](https://via.placeholder.com/150)

**Fig. 5.** Immunocytochemistry of stably transfected LLC-PK1 cell lines. Cells expressing M23-AQP4 (A) and M1-AQP4 (B) show basolateral staining with COOH-terminal anti-AQP4 antibodies. Peptide-depleted antibodies were used in (C) and (D) showing absence of membrane staining. Some nuclear staining was also seen in nontransfected LLC-PK1 cells and could represent a nuclear histone not related to AQP4 (20). Staining of the M1-AQP4 expressing cell membrane is more intense than in M23-expressing cells (see E and F for quantification). In M23 cells, the plasma membrane staining often takes the form of patches, possibly reflecting the presence of OAPs. The M1 staining pattern is more continuous and linear. Bar = 15 μm. Indirect immunocytochemistry of M23-AQP4 (A) and M1-AQP4 (B) was used to quantify AQP4 expression by fluorescence imaging. The mean pixel intensity of antibody-stained cell plasma membranes containing M1- and M23-AQP4 water channels was determined at various anti-AQP4 antibody dilutions. Graphic representations of antibody staining as a function of dilution are shown in (E). Regression analysis (E) of quantitative anti-AQP4 antibody staining (arbitrary units) as a function of antibody concentration (dilution) was based on values shown in F. Triple points at each dilution represent the mean value of four separate measurements and the means ± SD. The ratio M1/M23 = 7.5 was established in confluent and nonconfluent cultures.
immunocytochemically stained cells using a range of different antibody concentrations (Fig. 5, E and F). Importantly, OAPs were found only in the M23-AQP4-transfected cells (Fig. 6A). In contrast, OAPs were never found in cells expressing only M1-AQP4 (Fig. 6B). In addition, OAPs in M23-AQP4-expressing LLC-PK1 cells were larger than OAPs in native tissues (compare Fig. 6A with e.g., Figs. 2A and 3A).

To determine whether coexpression of M1- and M23-AQP4 in cell membranes could affect OAP organization, M1- and M23-expressing LLC-PK1 cells were seeded on the same culture dishes and were chemically fused by using polyethylene glycol. Stable somatic cell hybrids expressing both M1- and M23-AQP4 were obtained by selecting in medium containing hygromycin and Geneticin. Freeze-fracture examination of membranes from these stable cell lines revealed a reduced number of OAPs that were smaller than those found in M23-AQP4 cells (Fig. 6C).

Ser111 is a conserved amino acid among water-selective aquaporins, and in AQ4 it is part of a putative PKA (43) phosphorylation motif. To examine the potential role of this motif in OAP formation, stable LLC-PK1 cells were made expressing AQ4 point mutations in which the Ser 111 was replaced by glycine or glutamic acid. Freeze-fracture electron microscopic micrographs of M23-AQP4-Ser111G (not shown) and M23-AQP4-Ser111E (Fig. 6D) all displayed OAPs. The mean sizes of OAPs in micrometers squared were 0.030 ± 0.012 (wild-type; n = 28), 0.034 ± 0.011 (Ser111G; n = 14), and 0.071 ± 0.025 (Ser111E; n = 26), with similar expression levels to the wild-type M23 variant. Thus the Ser111E point mutations displayed significantly larger OAPs than wild-type or Ser111G M23-AQP4. The M1-AQP4 mutant counterparts were also made and expressed in LLC-PK1 cells. Similarly to wild-type M1-AQP4, they did not form OAPs (not shown).

Enhanced water permeability of cells expressing M23-AQP4 compared with M1-AQP4. Functionally, using video-enhanced DIC microscopy to visualize individual cells (Fig. 7, D and E), AQ4-expressing cells had a seven times higher \( P_f \) (Fig. 7, A and B, Table 1) than control cells (Fig. 7C). The volume increase of cells (calculated from quantitative DIC-microscopy measurements of cell radius) induced by hyposmotic DMEM was transient, probably reflecting the presence of volume regulatory responses. Interestingly, M1-AQP4 cells were significantly smaller, and M23-AQP4 cells were significantly larger in size than control LLC-PK1 cells (Fig. 7, D and E, Table 1). With \( P_f \) being equal (Fig. 7F), volume flow (Jv) was 2.5 times higher in M23-AQP4-expressing cells than in M1-AQP4-expressing cells. This is seen as a difference in amplitude of the volume traces (Fig. 7, A vs. B), as a result of the size difference.

Inspection of immunoblots also indicated higher expression levels of M1-AQP4 than M23-AQP4 when extracts of whole cells were utilized (Fig. 8, A and B). Densitometric analysis of anti-AQP4 staining of the 34-kDa band (Fig. 8A, top) and 31-kDa band (Fig. 8B, top), normalized to β-actin (Fig. 8, bottom) revealed a sixfold difference in expression (M1 > M23), consistent with the quantitative immunofluorescence data (Fig. 5), suggesting single-channel \( p_f \) is much greater for the M23 variant.

In the hybrid cell line, a three- to fourfold greater expression of M23-AQP4 than M1-AQP4 (Fig. 8C) was found. The \( P_f \) value was 1.5-fold greater than the \( P_f \) values in M1 and M23 cells (Fig. 7F, Table 1). Estimation of single-channel \( p_f \) by normalizing M23 expression in the hybrid cell line to AQ4 expression in M1 or M23 cells gave a value that was not significantly different from M1-AQP4 expressed alone.

M23-AQP4 cells expressing the Ser111E mutant were considerably smaller than cells expressing the wild-type M23 variant. The \( P_f \) value was 0.028 cm/s (Table 1), and expression levels were similar to AQ4 in M23 wild-type cells (not shown). Calculation of relative single-channel \( p_f \) in these cells gave a value that was >10-fold higher than M1-AQP4.

![Fig. 6. Freeze-fracture electron microscopy of stably transfected LLC-PK1 cell lines. A: M23-AQP4 cell membranes contain OAPs in basolateral P face. B: M1-AQP4 cell membranes do not contain OAPs. C: M1- and M23-AQP4 fused cells show fewer and smaller OAPs than cells expressing M23-AQP4 alone. D: M23-AQP4- Ser111E mutant with large OAPs in P-face view. Note the twofold greater area of OAPs in M23-AQP4 Ser111 mutants (D) when compared with wild-type M23-AQP4 (A). Bar = 100 nm. OAPs are contoured to enhance recognition.](https://www.ajprenal.org/content/287/SEPTEMBER/2004/F507)
These data indicate that the $p_f$ of OAP-forming M23-AQP4 is significantly greater than that of the non-OAP-forming M1-AQP4 but that it is not significantly different from M1-AQP4 when it is not arranged into OAPs.

**DISCUSSION**

These studies extend our previous observation that AQP4 does not always form OAPs in native cell plasma membranes and in stably transfected LLC-PK1 cells. Overexpression of M1 and M23 isoforms in LLC-PK1 cells established that M23-AQP4 is the OAP-forming water channel, whereas M1-AQP4 alone does not form OAPs. It is possible, therefore, that the relative levels of M1- and M23-AQP4 isoform expression could regulate OAP formation at the cell and tissue level in vivo. Thus M1-AQP4 should be the predominantly expressed isoform in gastric tissues of mouse and kangaroo rat, as well as in mouse proximal tubules [which express abundant AQP4 but only rare OAPs (38)], whereas M23-AQP4 should be predominant in Sprague-Dawley and Brattleboro rat parietal cells. However, Western blot analysis indicated that M23-AQP4 is always the most abundant protein in tissues (16, 38), suggesting that small changes in M1-AQP4 content may be sufficient to modify the size of OAPs. Support for this idea comes from our somatic cell hybrids in which expression of the M23 isoform is three- to fourfold greater than the M1 isoform, yet these

Fig. 7. Functional analysis of AQP4 in stably transfected LLC-PK1 cells. M23-AQP4 (A), M1-AQP4 (B), and control (C) cells were perfused with hypotonic and isotonic solutions at room temperature, and images were captured every second by video-enhanced differential interferential contrast (DIC)-microscopy. Upward arrows indicate when DMEM solutions at indicated osmotic strengths were administered, resulting in rapid changes in volume. M23-AQP4-expressing cells (A), (initial volume 13,000 µm$^3$) exhibited relatively large volume changes compared with M1-AQP4 (B) (initial volume 6,000 µm$^3$) but have similar half-times. Control LLC-PK1 cells (C) had a relatively low rate of osmotic water permeability and had an initial volume of (9,000 µm$^3$). M23-AQP4 (D) and M1-AQP4 (E) cells seen by DIC-microscopy consistently showed a difference in cell size, with M23-expressing cells being larger.

$P_f$ bar graph of osmotic water permeability coefficients ($P_f$) depicts the mean and SE. Student’s $t$ tests indicate significant differences (**$P < 0.001$) between control LLC-PK1 cells and M1 and M23-AQP4 transfected cells. In addition, $P_f$ values of somatic cell hybrids (expressing both M1- and M23-AQP4) or the M23-Ser$^{111E}$ mutants were significantly different from values of M1 or M23 cells (*$P < 0.025$).
cells contain fewer and smaller OAPs than cells in which the M23 isoform is expressed alone.

Functional studies using a cell-swelling assay and quantification of plasma membrane AQP4 content indicates an eightfold higher single-channel water permeability of M23-AQP4 compared with M1-AQP4. This difference may originate from the geometric arrangement of AQP4 monomers into OAPs. Consequently, disruption or dispersion of M23-AQP4 might reduce $P_f$ to a value comparable to that of M1-AQP4. One caveat when calculating a single-channel $P_f$ is the presence of “nonfunctional” but antigenically detectable channels of AQP4 on the membrane leading to underestimation of single-channel $P_f$. Another caveat is when M23-AQP4 is organized into OAPs shielding the epitope for antibody binding, causing an overestimation of single-channel $P_f$. However, epitope shielding may be excluded from these considerations, because Western blot analysis of denatured and soluble protein gave similar results obtained from immunocytochemistry. Our data differ from those obtained in a Xenopus oocyte-based assay (29). Oocytes injected with different ratios of M1 and M23 cRNA exhibited comparable $P_f$ rates and expressions. Given the low level of M1-AQP4 expression in native tissues (16, 29, 38), (i.e., M1/M23 $\ll$ 0.25), higher M1/M23 ratios ($>0.25$) expressed in oocytes may not be compatible with OAP formation. When M23-AQP4 was the only water channel expressed in oocytes, the protein level was not reported, making it difficult to determine the relative single-channel permeability of the M1 vs. the M23 variants alone. We also found identical $P_f$ values, but expression levels of M23-AQP4 compared with M1-AQP4 in LLC-PK$_1$ cells were significantly lower, and the size of LLC-PK$_1$ cells expressing M23-AQP4 was significantly larger than M1-expressing cells (see below). However, it is also possible that M23-AQP4 in oocytes did not form OAPs, or that in stably transfected LLC-PK$_1$ cells there may be an upper limit of measurable osmotic water permeability when cells are overexpressing a water channel, which would result in the identical $P_f$.

![Image](http://ajprenal.physiology.org/)

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**Table 1. Properties of M1- and M23-AQP4-expressing LLC-PK$_1$ cells**

<table>
<thead>
<tr>
<th></th>
<th>$P_f$, cm/s</th>
<th>$n$</th>
<th>Radius, μm</th>
<th>OAP Size, μm$^2$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0031 ± 0.0005</td>
<td>9</td>
<td>9.2 ± 0.46</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>M1-AQP4</td>
<td>0.0188 ± 0.0012</td>
<td>17</td>
<td>7.3 ± 0.13</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>M23-AQP4</td>
<td>0.0187 ± 0.0013</td>
<td>16</td>
<td>11.2 ± 0.21</td>
<td>40</td>
<td>0.030 ± 0.0023</td>
</tr>
<tr>
<td>M1/M23</td>
<td>0.0283 ± 0.0037</td>
<td>13</td>
<td>11.9 ± 0.43</td>
<td>13</td>
<td>0.002 ± 0.0004</td>
</tr>
<tr>
<td>Ser$^{111E}$</td>
<td>0.0314 ± 0.0055</td>
<td>11</td>
<td>8.9 ± 0.27</td>
<td>11</td>
<td>0.071 ± 0.0049</td>
</tr>
</tbody>
</table>

Values are means ± SD. Osmotic water permeability coefficient ($P_f$) values, cell radii, and sizes of orthogonal array particle (OAP) aggregates were measured as described in MATERIALS AND METHODS. $n$ is the number of cells subjected to $P_f$ measurements, radii measurements, or number of OAPs found on representative replicas from different experiments. Student’s $t$-tests showed significant differences in $P_f$ values between control and M1 or M23 cells ($P < 0.001$), between M1 and M23 cells vs. M1/M23 somatic cell hybrids or M23-Ser$^{111E}$ aquaporin-4 (AQP4)-expressing cells ($P < 0.025$). Significant differences in radii were found between cells expressing M1 vs. M23 isoforms, between M1 and M23 cells vs. control ($P < 0.001$), and between control and the M1/M23 hybrid cells ($P < 0.001$). Significance was absent when radii of control cells were compared with radii of M23- Ser$^{111E}$ mutants. OAP sizes of wild-type M23-AQP4, Ser$^{111E}$-M23 mutant, and M1/M23 hybrids were significantly different from each other ($P < 0.001$).

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**Fig. 8.** Immunoblots of stably transfected LLC-PK$_1$ cell lines. A dilution series of cell extracts of M1-AQP4 (A), M23-AQP4 (B), and M1/M23-AQP4 (C) expressing LLC-PK$_1$ cells on Western blots were stained with anti-AQP4 antibodies (top), and anti-β-actin antibodies (bottom). Lane 1 contained -90 μg (A), 70 μg (B), and 5 μg protein (C). Lanes 2-5 contained 1/4, 1/16, 1/64, and 1/256 of the indicated amount of protein.

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values measured here. Selection pressure probably plays a critical role when LLC-PK1 cells are passaged and propagated. It is possible that a very high water permeability coefficient makes the cell fragile, and mechanical challenges during passage could be deleterious. Thus only cells with a limited permeability coefficient would survive. With respect to the somatic hybrid cell line, showing a much higher AQP4 content of the M23 variant with a limited number of OAPs that were smaller in size, the average single-channel water permeability coefficient of M23-AQP4 was reduced. This would allow enhanced M23 expression up to the hypothesized upper limit of membrane P0.

In addition to a role of the different AQP4 isoforms in OAP formation, we also found that changes in AQP4/OAP morphology were produced by chronic vasopressin treatment of Brattleboro rats. In collecting duct principal cells, chronic dDAVP treatment induced the appearance of irregular IMP-aggregates at the apparent expense of OAPs on the P face of the plasma membrane, whereas E-face views maintained typical checkerboard impressions of OAPs. This suggests an asymmetrical transmembrane rearrangement of AQP4 molecules that at present is of uncertain functional relevance. The fact that a similar arrangement was not seen after acute (several hours) dDAVP exposure implies that the rearrangement might not be a direct effect of the hormone, but might result from a progressive increase in interstitial osmolality that occurs in dDAVP-treated Brattleboro rats (12).

One possibility, however, is that the potential PKA phosphorylation site at Ser111 in AQP4 could be involved in OAP formation. Indeed, the ability of M23-AQP4 to form OAPs was increased 2.5-fold when Ser111 was mutated to glutamic acid, an amino acid that mimics constitutive phosphorylation of this residue. However, OAP formation remained similar to the wild-type formation when Ser111 was replaced by glycine. Whether the previously described short-term effect of dDAVP to increase OAP formation (28, Fig. 3B) reflects Ser111 phosphorylation will require further detailed studies. The effect of chronic dDAVP administration on perturbation of OAP organization could be a reflection of a complex pattern of secondary regulatory effects, including increased medullary hypertonicity. Other kinases also play a role in AQP4 biology. Phosphorylation of AQP4 by casein kinase II has been reported to enhance lysosomal targeting and degradation of AQP4 and therefore to regulate AQP4 cell surface expression in MDCK cells (24). PKC and dopamine decrease AQP4 water permeability via phosphorylation at Ser180 and the effect is probably mediated by gating of the channel (11, 46). Conformational changes of AQP4 and OAPs were not examined in this study, however. Intracellular cAMP levels and subsequent activation of PKA in gastric glands have been reported also (5, 9, 13, 36). The role of AQP4 in parietal cells is unclear, but HCl secretion by these cells may be associated with water transport to produce a secreted fluid that is isotonic with plasma. Thus AQP4 may be subject to different kinds of phosphorylation, which could possibly contribute to OAP aggregation and OAP disruption in kidney and gastric glands.

Finally, a consistent finding in our LLC-PK1 clones transfected with M1- and M23-AQP4 was that cell size was significantly increased by M23-AQP4 overexpression, and decreased by M1 overexpression. Interestingly, a reduction in cell size in astrocytes by RNA silencing of AQP4 was recently reported (30). Whether this effect is related to osmoregulatory properties of the plasma membrane after AQP4 expression or some other as yet unidentified cellular function of AQP4 remains to be determined.

Our present data indicate (1) that AQP4 protein can be expressed in cell membranes in various morphological configurations that are cell type, species, and hormone dependent; (2) that M23-AQP4 forms OAPs but M1-AQP4 does not; (3) that the M1-AQP4 variant could regulate OAP structure; and (4) that M23-AQP4 in OAPs has a greater single-channel water permeability than M1-AQP4.

While this article was in the final stages of manuscript preparation, an article appeared (7) also showing that M23-AQP4 in stably transfected CHO cells, but not M1-AQP4, is the OAP-forming isoform of AQP4. These results on nonpolarized cells are in complete agreement with our present data obtained by using a kidney epithelial cell line.

GRANTS

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