Immunocytochemical Localization of Aquaporin-1 in Bovine Corneal Endothelial Cells and Keratocytes

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For immunocytochemistry, cultured bovine corneal endothelial cells (CBCEC) and bovine corneal cryosections were utilized. Preparations were fixed, permeabilized, and incubated with primary rabbit anti-rat aquaporin 1 (AQP1) antibody followed by rhodamine-conjugated secondary antibody, and were counterstained with Sytox nuclear acid stain. Confocal microscopy of CBCEC in the x, y, and z planes showed rhodamine fluorescence, indicating the presence of AQP1 antibody localized to the apical and basolateral domains of the plasma membrane, but not to the membranes of intracellular compartments or other subcellular locations. Preabsorption with control antigenic peptide yielded no positive staining. Similar results were obtained using freshly dissected bovine corneas; in addition, these images showed AQP1 distributed to the plasma membranes of keratocytes. No AQP1 staining was seen in corneal epithelium, and no staining was observed in CBCEC layers exposed to AQP3, AQP4, and AQP5 antibodies. [Exp Biol Med Vol. 226(5):463–467, 2001]

Key words: antibody; fluid transport; water channel; confocal microscopy; apical; basolateral

Water crosses plasma membranes either by diffusion across the lipid bilayer or through water channel proteins known as aquaporins (AQPs). One or more AQP isoforms is present in every fluid-transporting epithelial layer investigated so far. On the other hand, less is known about the intracellular distribution of AQPs. In one important case, that of the kidney collecting duct, the AQP distribution is polarized, with AQP2 being inserted apically upon vasopressin stimulation (3, 13), and AQP3 being constitutively expressed in the basolateral membranes (6). However, not every epithelium is equally well characterized with respect to these water channel proteins.

For the cornea, the presence of three isoforms has been described (AQP1, AQP3, and AQP5) (11), two of them (AQP3 [4] and AQP5 [12]) being found in corneal epithelium, and AQP1 being found in stromal keratocytes (4) and corneal endothelium (5, 8). In particular, the corneal endothelium is the site of a fluid transport mechanism that is essential to maintain normal corneal hydration, and thus transparency. Although the precise mechanism by which fluid transport occurs is still unknown, it is presumed that passage of fluid across AQP1 is an important component of it. Yet, the possible presence of other AQP isoforms in the endothelium has not been excluded. In addition, the distribution of the known isoform present (AQP1) to the different membrane domains of the endothelial cells has not previously been described. We undertook this immunocytochemical study to attempt to clarify such issues. We found that AQP1 is present throughout all plasma membrane domains (apical, lateral, and basal) of cultures bovine corneal endothelial cells (CBCEC). We found no evidence for the presence of AQP3, AQP4, or AQP5.

Materials and Methods

Cell Culture. CBCEC were cultured and subcultured as described previously (1). Briefly, bovine eyes maintained in ice were obtained from a local abattoir some 7 hr after the death of the animals. On arrival, each eye was wiped with alcohol for sterilization. The cornea was removed, placed in a shallow hemispherical holder (endothelium up), and washed with a calcium-free physiological buffer solution. The endothelium was then covered with 1 ml of calcium- and magnesium-free saline solution containing 0.25% trypsin and 0.02% ethylene-diamine-tetraacetic acid (EDTA), and the holder was placed in an incubator for 5 to 10 min.

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The solution then was changed to Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY), and the endothelium was gently rubbed with a plastic rod to loosen the cells. The cells were aspirated and plated in 25-cm^2 culture flasks (Falcon; Becton Dickinson, Franklin Lakes, NJ) with 3 ml of DMEM supplemented with 6% heat-inactivated fetal bovine serum, 2 ng/ml basic fibroblast growth factor, and antibiotics (penicillin 100 U/ml and streptomycin 2.5 μg/ml). Cells were maintained in a CO_2 incubator and were provided with new culture medium every 3 days.

Cells reached confluence in 5 to 7 days, after which they were subcultured. Culture medium was aspirated, and 1 ml of trypsin-EDTA solution was added. The cells were then observed under a microscope; when they began to round up, fresh DMEM containing 6% (v/v) fetal bovine serum was added. Cells were loosened from the flask by gentle trituration by cyclic aspiration into and emptying of a 10-ml pipette. The cell suspension was divided into four flasks (25 cm^2, as above) and grown to confluence. For the experiments, one flask of cells was subcultured into up to eight 2-well chamber slide systems (Nunc, Naperville, IL).

Corneal Tissue Preparation. Bovine eyes were obtained from a slaughterhouse as described above. Prior to dissection, the ocular globes were washed with phosphate-buffered saline (PBS, Gibco-BRL). The globes were placed in a Petri dish, and the corneas were dissected off. Corneas were transferred to a dish, were washed twice with HEPES-buffered Hank's balanced salt solution (Medium 199, Gibco-BRL), and were cut in rectangular pieces of about 1 × 0.5 cm. Such pieces were subsequently washed three times with medium 199 at 37°C, and were then fixed as described below.

Immunocytochemistry. CBCEC subcultured as above were washed twice with medium 199 at 37°C, fixed for 30 min in PLP fixative (9) (2% formaldehyde, 75 mM lysine, 10 mM sodium periodate, and 45 mM sodium phosphate, pH 7.4), washed in PBS three times, permeabilized with 0.075% saponin in PBS for 20 min, and washed in PBS three times. The fixed and permeabilized cell monolayers were then incubated in blocking solution (15% goat serum, 0.3% Triton X-100, 20 mM sodium phosphate, and 0.9 mM sodium chloride) for 30 min. After removing the blocking solution by aspiration, the monolayers were exposed to rabbit anti-rat AQP1 antiserum (category no. AQP11-A, Alpha Diagnostic International, San Antonio, TX [ADI]) diluted in PBS plus 15% goat serum, 0.2% bovine serum albumin (Sigma, St. Louis, MO) in PBS. After some tries, we settled on a dilution of 1:500 for this primary antibody (final concentration of 2 μg/mL). As a control we preabsorbed with the immunogenic AQP1 C-terminal peptide used to generate the antibody (251–269; EE YDL DAD DIN SRV EMK PK; category no. AQP11-P, ADI; final concentration of peptide: 2 μg/mL, in PBS). The incubations were done for 60 min in a humid chamber at room temperature. Subsequently, the cell layers were washed three times in PBS and twice in PBS plus 5% goat serum. Then the cell layers were incubated in Rhodamine Red-X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:200 (in PBS) for 90 min in the humid chamber at room temperature. Samples were prepared and kept in the dark to prevent light-induced damage to Rhodamine Red-X.

Corneal tissue samples were fixed in PLP medium for 1 hr, and were washed in PBS three times. They were then immersed in 30% sucrose and kept overnight at 4°C. Subsequently, the excess solution was absorbed away, and the pieces were embedded in O.C.T. (Optimal Cutting Temperature compound, Tissue-Tek, Sakura Finetek, Torrance, CA). The pieces were then stored in a −20°C refrigerator and were subsequently sliced in a cryostat (CM 1850, Leica Microsystems, Bannockburn, IL; 4-μm-thick specimens). Specimens were collected on Silane-Prep slides (Sigma, St. Louis, MO). After this, specimens were permeabilized with 0.075% saponin in PBS for 20 min and were washed in PBS three times. The rest of the procedure is the same as described for the cultured cells above. For control experiments we used the antigen peptide.

Nuclear Counterstaining and Mounting. Samples were equilibrated briefly in 2× SSC solution (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0, titrated with HCl), and were then incubated for 20 min in 2× SSC solution containing 5 μg/ml DNAse-free RNAse (Boehringer Mannheim, Indianapolis, IN). Incubation was terminated with three washes in 2× SSC solution (1 min each), after which samples were incubated for 5 min in Sytox Green (Molecular Probes, Eugene, OR) diluted 1:300 (v/v) in 2× SSC solution, and were then rinsed five times (1 min each) in 2× SSC solution. The upper part of the chamber slide system was then quickly removed, leaving only the cells on the slides. Finally, coverslips were applied to the samples using one drop per well of H-1000 Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). The excess mounting medium was aspirated, and the coverslips were secured with clear nail polish.

The preparations were screened for fluorescence with a Axiovert S100 microscope (Carl Zeiss, Thornwood, NY) using excitation wavelengths of 480 and 545 nm to detect emission by nuclear staining (Sytox Green) or by antibody staining (Rhodamine Red-X), respectively. Preparations showing staining were then examined with a scanning confocal microscope (LSM 410, Carl Zeiss). Excitation came from its argon-krypton laser producing lines at 488 or 568 nm. Fluorescence in the x, y plane was recorded at different depths, and fluorescence in the x, z and y, z planes was obtained from the combined x, y images using Zeiss LSM-PC software. The images were enhanced using Adobe Photoshop software (San Jose, CA).

Results

Figures 1 through 3 show results that are representative of 11 wells of cultured cells that were analyzed by confocal

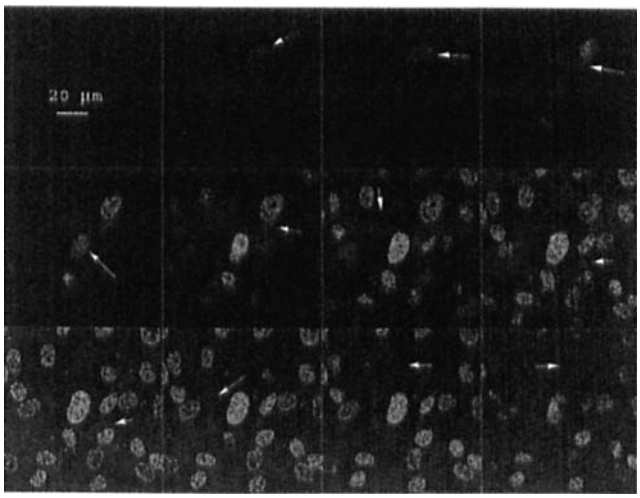

Figure 1. Immunofluorescence localization of AQP1 in CBCEC. Serial confocal optical sections extending from a plane just above the apical membrane to the basal membrane (left to right; top to bottom). Red, labeling with AQP1 antibody and rhodamine-conjugated secondary antibody. Green, Sytox green-stained nuclei. AQP1 is in the apical membranes, which are rhodamine-stained (frames 2–5), as well as in the lateral ones (frames 6–10). There is also some suggestion (frames 11 and 12) of basal staining. Arrows point to the features noted.

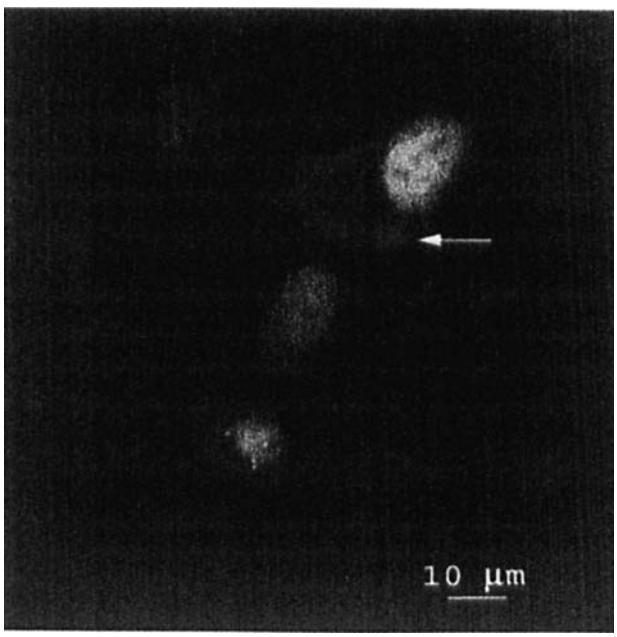

Figure 2. Enlargement of frame 4 in Fig. 1. The arrow points to the staining of the apical membrane.

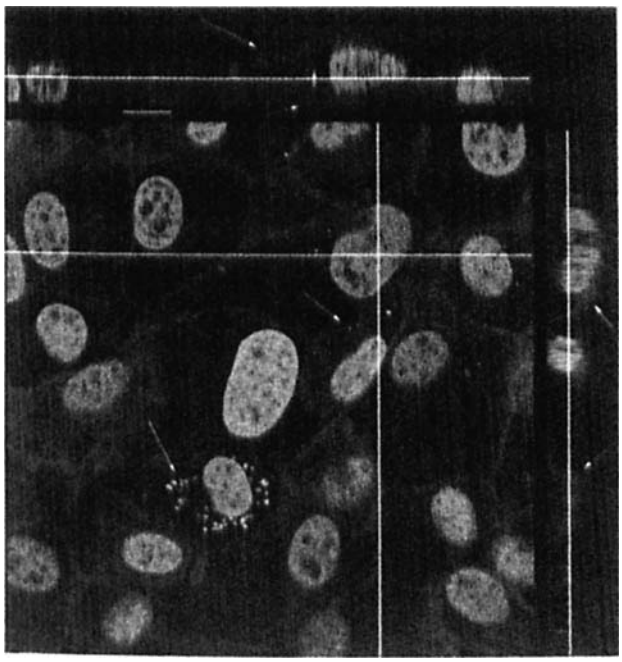

Figure 3. The large square panel (side length: 101.5 µm) presents a cross section of the cell layer which corresponds to frame 8 of the gallery in Fig. 1. The horizontal and vertical white lines through it denote the planes of the adjoining x, z and y, z sections, respectively. At the top and right, the x, z and y, z cross sections shown were obtained from the combined images in Fig. 1 using Zeiss LSM-PC software. White lines through them denote the depth of the relevant z plane. Marker atop the large panel, 10 µm. Arrows point to the AQP1 staining, except for arrow at left bottom, which points to Sytox green-stained RNA elements in a cell on which RNAase apparently had little effect.

microscopy. Optical sections showed rhodamine fluorescence indicating the presence of AQP1 antibody predominantly localized to the plasma membrane, rather than to membranes of intracellular compartments or other subcellular locations. Figure 1 shows a gallery of 12 optical sections, starting with a section located just above the apical cell membrane, with subsequent sections continuing stepwise towards the basal membrane. In this gallery, images 2 and 3 show a spot of Rhodamine Red-X staining, representing the apical membrane above the nucleus of a cell bulging slightly upwards. The next gallery images (4 and 5) show the first sections through the top of the nucleus, stained with Sytox Green, and a part of the apical membrane sloping down along the nucleus and also stained in red. Images 7 to 11 of the gallery show clearly the outline of the lateral membrane domain stained with Rhodamine Red-X. Image 4 of the gallery is shown in enlarged form in Fig. 2 to show in detail the top of the nucleus and the adjacent apical membrane and its staining. In the same vein, image 7 is shown enlarged in Fig. 3 (lower left quadrant of Fig. 3) to demonstrate the staining of the lateral membrane domain more clearly.

Figure 3 shows a three-dimensional reconstruction of the AQP1 antibody distribution corresponding to the Rhodamine Red-X fluorescence. The antibody location at the apical and basal membranes is particularly well apparent in the z-sections, as well as the lateral location in the z-sections and main panel. Control experiments performed with the addition of antigenic peptide (data not shown) showed no counterstaining with Rhodamine Red-X.

Figure 4 shows the expression and distribution of AQP1 in freshly dissected bovine corneal endothelium. As can be seen, the distribution is the same as that seen in CBCEC. The cross section gives a clear picture confirming that AQP1 is clearly present in both apical and basolateral membrane domains.

It is also interesting to verify that the information in Fig. 4 confirms the presence of AQP1 in keratocytes, as reported earlier (4). The gallery of optical slices in Fig. 5 shows this in more detail; as can be seen there, AQP1 is expressed in the plasma membrane and is distributed all around the keratocytes. In contrast, no staining for AQP1 was detected in corneal epithelium (not shown).

Lastly, in another series of experiments we exposed CBCEC layers to AQP3, AQP4, and AQP5 antibodies (category nos. AQP31-A, AQP41-A, and AQP51-A; ADI) using the same protocol as was used with AQP1 antiserum. In none of these experiments was any staining of membrane domains observed (data not shown).

Discussion

CBCEC layers grown on permeable supports have been shown to transport fluid (10) at rates comparable with those seen in freshly excised rabbit corneal layers. Furthermore, sodium, potassium, two chloride cotransporters (NKCC) have been reported to be similarly distributed to the basolateral membranes of CBCEC and fresh rabbit and bovine corneal endothelial cells grown on impermeable supports (7). From this we surmise that CBCEC are polarized in the same manner as their counterparts in animal tissues, and that they represent a good model for functional as well as morphological studies.

To date, at least nine AQP (AQP1–9) have been identified in animals, plants, and microorganisms, five of them (AQP1–5) in mammalian tissues. In the cornea, as mentioned above, AQP 1, 3, and 5 are known to be present. AQP3 and AQP5 have been localized to the corneal epithelium (4, 12); our evidence indirectly confirms that location, in that we could not detect them in CBCEC.

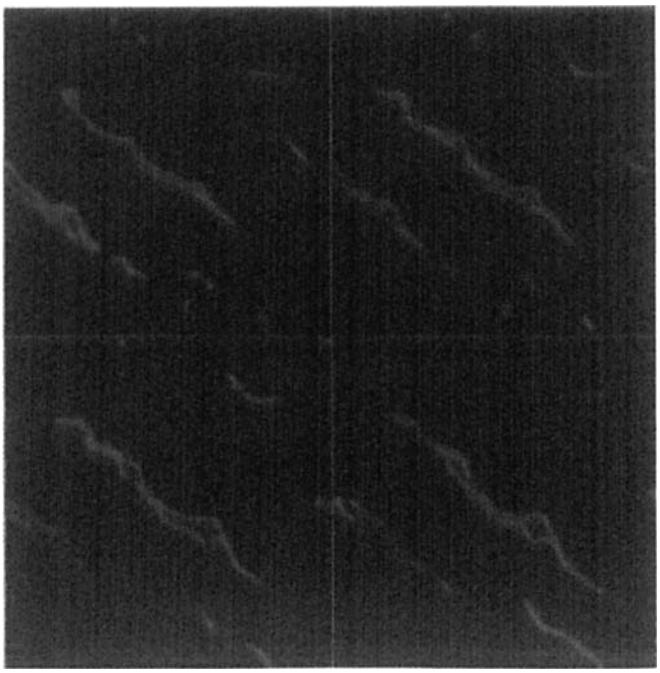

Figure 4. Section across bovine cornea depicting the corneal endothelium plus a segment of underlying Descemet's membrane and stroma. The length of the (square) sides of the picture is 48 μm. For clarity, samples were scanned only for red fluorescence depicting AQP1 staining; nuclear staining was also visible (not shown). The arrow points to the apical side of an endothelial cell. AQP1 staining of underlying keratocytes is also apparent.

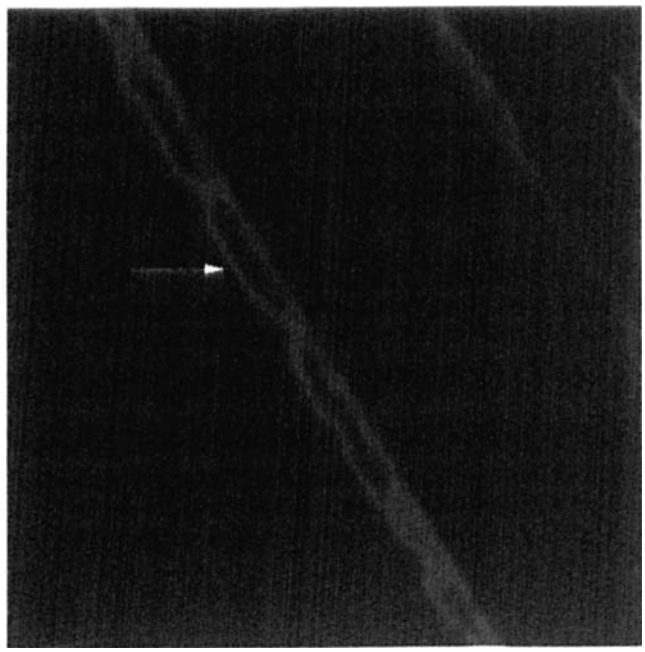

Figure 5. Gallery of sequential optical slices (depth: 1 μm; top to bottom, left to right) across a piece of bovine cornea, depicting the distribution of AQP1 in keratocytes. As in Fig. 4, samples were scanned only for red fluorescence depicting AQP1 staining; nuclear staining was also visible (not shown).

In a previous study of ours (8) we presented evidence that AQP1 was present in the plasma membrane of corneal endothelial cells. However, the evidence in that study did not allow one to determine to which plasma membrane domain(s) was AQP1 distributed. Such determination has become important to evaluate the possible validity of an oscillatory mechanism of transcellular fluid transport recently proposed (2, 14). For such mechanism to be feasible, we find that differential activation of apical and basolateral water channels is necessary (Iserovich P, Rubashkin A, Reinach PS, Hernández JA, Fischbarg J., unpublished data). However, the present study shows that AQP1 is distributed to both the apical and basolateral membrane domains of cultured and freshly excised bovine corneal endothelial cells. In addition, a prior report had shown (4) in less detail basolateral and apical AQP1 staining of rat corneal endothelial cells. Such findings, coupled with the absence of other aquaporins in corneal endothelium (4; this paper), raise doubts as to whether oscillatory transcellular fluid transport could take place, as this would require a complex modulatory process capable of discriminating between apical versus basolateral aquaporins.

In a prior report (4), a strong reaction of keratocytes with anti-AQP1 had been noted in rat and human corneas. Our images show more detail and reveal that this protein is present in the plasma membrane of the keratocytes. This appears intriguing. As for the physiological role of AQP1 in

this case, it might facilitate rapid volume response or deformation by such cells or might facilitate homogeneous osmotic water flows across them in the anteroposterior direction. To determine whether keratocytes may exhibit such characteristics will require further work.

As for the corneal endothelium, the location of AQP1 on both apical and basolateral membrane domains is consistent with cellular functions, including translayer osmotic fluid flows, volume regulation, or hypothetical transcellular route for fluid transport. Again, to determine whether any such role is predominant or exclusive also requires further work.

1. Diecke FPJ, Zhu Z, Kang F, Kuang K, Fischbarg J. Sodium, potassium, two chloride cotransport in corneal endothelium: Characterization and possible role in volume regulation and fluid transport. Invest Ophthalmol Vis Sci **39:**104–110, 1998.
2. Fischbarg J. Mechanism of fluid transport across corneal endothelium and other epithelial layers: A possible explanation based on cyclic cell volume regulatory changes. Brit J Ophthalmol **81:**85–89, 1997.
3. Fushimi K, Uchida S, Hara Y, Hirata Y, Marumo F, Sasaki S. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. Nature **361:**549–552, 1993.
4. Hamann S, Zeuthen T, La Cour M, Nagelhus EA, Ottersen OP, Agre P, Nielsen S. Aquaporins in complex tissues: Distribution of aquaporins 1–5 in human and rat eye. Am J Physiol **274:**C1332–C1345, 1998.
5. Hasegawa H, Zhang R, Dohrman A, Verkman AS. Tissue-specific expression of mRNA encoding rat kidney water channel CHIP28k by *in situ* hybridization. Am J Physiol **264:**C237–C245, 1993.
6. Ishibashi K, Sasaki S, Fushimi K, Uchida S, Kuwahara M, Saito H, Furukawa T, Nakajima K, Yamaguchi Y, Gojobori T, Marumo F. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. Proc Natl Acad Sci U S A **91:**6269–6273, 1994.
7. Jelamskii S, Sun XC, Herse P, Bonanno JA. Basolateral Na(+)-K(+)-2Cl(-) cotransport in cultured and fresh bovine corneal endothelium. Invest Ophthalmol Vis Sci **41:**488–495, 2000.
8. Li J, Kuang K, Nielsen S, Fischbarg J. Molecular identification and immunolocalization of the water channel protein Aquaporin 1 in CB-CECs. Invest Ophthalmol Vis Sci **40:**1288–1292, 1999.
9. McLean IW, Nakane PK. Periodate-lysine-paraformaldehyde fixative: A new fixation for immunoelectron microscopy. J Histochem Cytochem **22:**1077–1083, 1974.
10. Narula PM, Xu M, Kuang K, Akiyama R, Fischbarg J. Fluid transport across cultured bovine corneal endothelial cell monolayers. Am J Physiol **262:**C98–C103, 1992.
11. Patil RV, Saito I, Yang X, Wax MB. Expression of aquaporins in the rat ocular tissue. Exp Eye Res **64:**203–209, 1997.
12. Raina S, Preston GM, Guggino WP, Agre P. Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal and respiratory tissues. J Biol Chem **270:**1908–1912, 1995.
13. Sabolic I, Katsura T, Verbavatz JM, Brown D. The AQP2 water channel: Effect of vasopressin treatment, microtubule disruption, and distribution in neonatal rats [published erratum appears in J Membr Biol **145**(1):107–108, 1995]. J Membr Biol **143:**165–175, 1995.
14. Walker VE, Stelling JW, Miley HE, Jacob TJC. Effect of coupling on volume-regulatory response of ciliary epithelial cells suggests mechanism for secretion. Am J Physiol **276**(Cell Physiol. 45):C1432–C1438, 1999.