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Immunolocalization of Aquaporin Homologs in Mouse Lacrimal Glands

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It was shown recently that the aquaporin family of water channels exists in lacrimal gland cells. To determine the localization of aquaporin homologs and the pathophysiological modification of aquaporins by pilocarpine, a muscarinic agonist, we performed immunohistochemistry and immunoblotting in mouse lacrimal glands. By immunohistochemistry, aquaporin-4 (AQP4) and aquaporin-5 (AQP5) were found to be the lacrimal glands. AQP5 immunolabeling was detected in the apical membranes of the acinus and duct cells, while AQP4 was expressed in the basolateral membranes only. The tear secretion of mice systemically treated with pilocarpine was significantly (2.5-fold) higher than that of the saline-treated controls. The antibody to the AQP5 carboxy terminus showed high immunoreactivity on the apical membrane in the pilocarpine-treated lacrimal glands but not on that of saline-treated controls. However, the antibody to the extracellular domain of AQP5 showed similar immunolabeling in both groups of animals. In contrast, the immunoreactivity of AQP4 was not affected by pilocarpine stimulation. As shown by western blot analysis, the expression level of AQP5 on the apical membrane in the pilocarpine-stimulated lacrimal glands was not significantly different compared with the saline-treated controls. We conclude that AQP4 and AQP5 water channels are expressed on mouse lacrimal gland cells, with greater expression of AQP4 on the basolateral membrane and of AQP5 on the apical membrane. Furthermore, the AQP5 carboxy terminus region may undergo pathophysiological modification when tear secretion is increased by pilocarpine stimulation.

Lacrimal glands transport fluid for secretion as tears. Two types of tear secretion were postulated, basic and reflex (1). Basic secretion is thought to be a constant and slow secretion. Reflex secretion is defined as rapid secretion caused by neural stimulation and is thought to occur primarily in the main lacrimal glands. These lacrimations are regulated by the autonomic nervous system and several hormones. Fluid movement through glandular cells is thought to be caused by several transporters in the cell membrane and depends upon the active transport of ions (2). However, the molecular mechanism of the water transport has not yet been completely identified. Recently, functional water channels have been identified in mammals, and this group of proteins has been referred to as the "aquaporins" (3). These proteins are expressed in secretory tissues and glands, suggesting that the water channel family is essential for a rapid water transport. We recently obtained the functional evidence of the presence of aquaporin-5 (AQP5) water channels in the lacrimal gland, i.e. *Xenopus* oocytes injected with rat lacrimal gland poly(A)$^+$RNA showed an increase in water permeability that was abolished by an AQP5 antisense oligonucleotide (4). In addition, northern and *in situ* hybridization analyses indicated the distribution of the AQP5 and AQP4 transcripts in lacrimal glands (5, 6). These AQP water channels are thus believed to play a key role in transcellular water transport for lacrimation.

Based on amino acid identity, AQP5 is more closely related to aquaporin-2 (AQP2) than to the other aquaporins (6). The AQP2 molecule is translocated from the intracellular vesicle to the apical membrane by vasopressin stimuli, resulting in an increase in water permeability through the plasma membrane for urine concentration (7). AQP5 may also be under similar regulatory controls, contributing to tear secretion. The purpose of this study was to examine the membrane localization of mouse lacrimal gland water channels by immunohistochemistry and to confirm whether the distribution of aquaporin proteins in glandular cells is changed by inducing an increase in tear secretion as occurs with AQP2 regulation.

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Abbreviations used: AQP, aquaporin; PBS, phosphate buffered saline.

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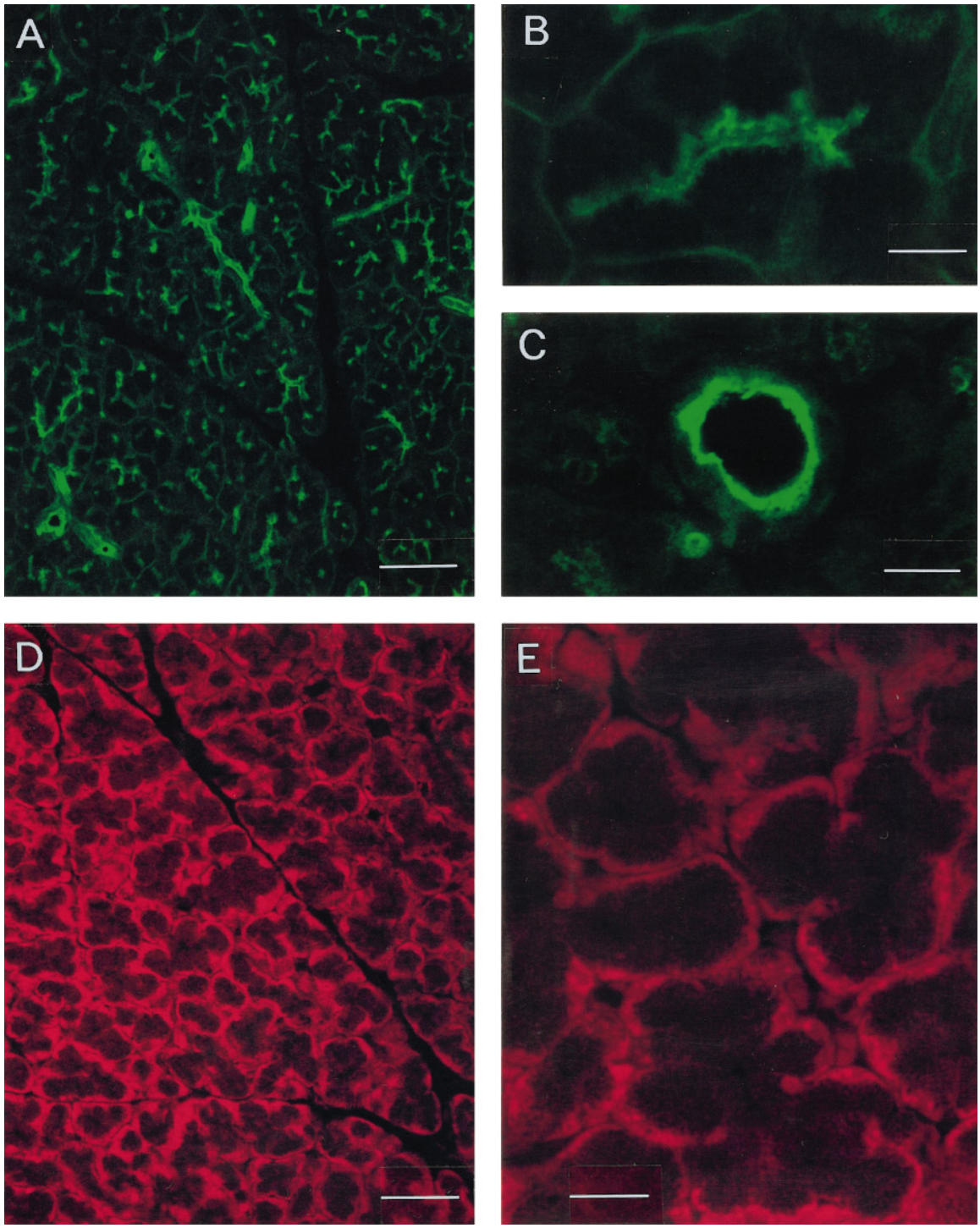
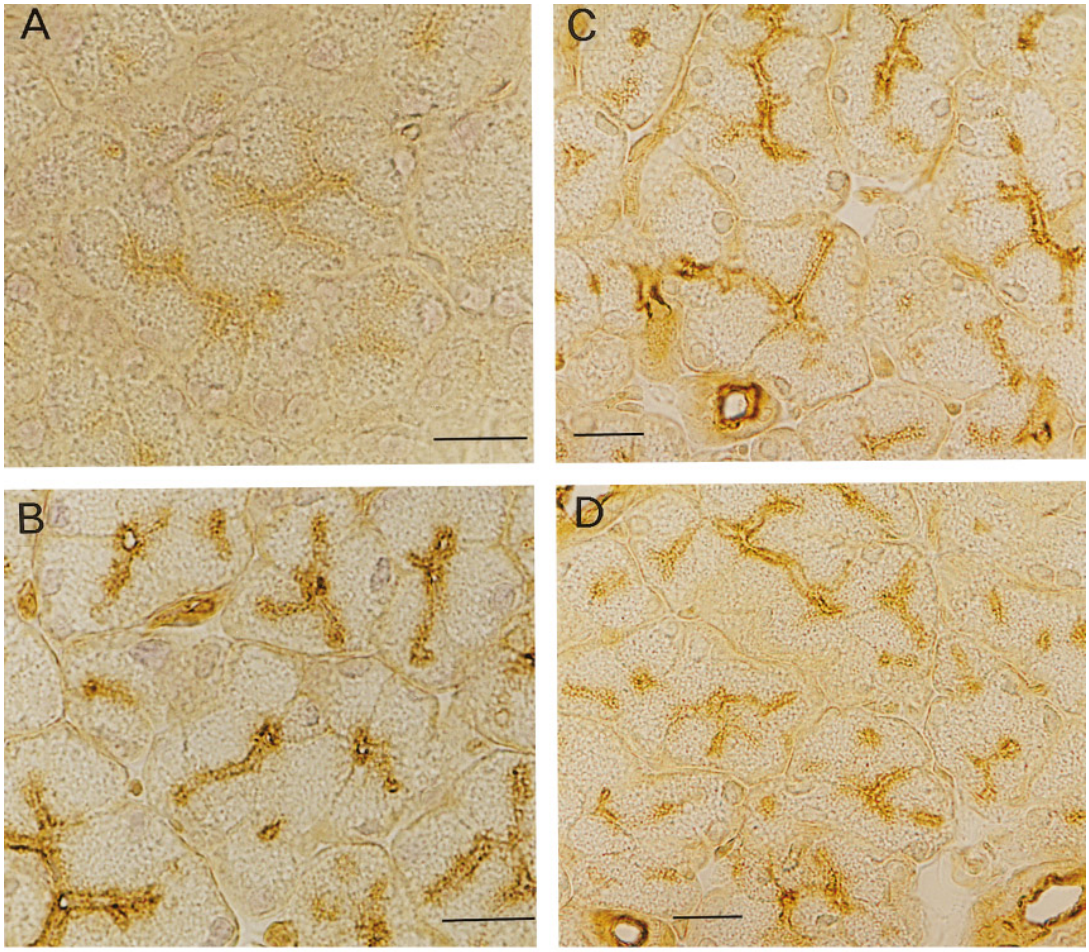


FIG. 1. Immunofluorescence of the mouse exorbital lacrimal gland using purified AQP5 and AQP4 polyclonal antibodies. (A) Low magnification immunofluorescence (FITC label) of the lacrimal gland stained with purified AQP5 antibody to the extracellular domain. (B and C) High magnification AQP5 immunostaining shows the localization on the apical membrane of the acinus (B) and duct (C) cells. (D) Low magnification immunofluorescence (rhodamin label) of the lacrimal gland stained by AQP4 antibody. (E) High magnification microscopy shows the AQP4 immunolabeling on the basolateral membrane of the acinar cells. Bars : A and D, 50 μm; B,C,E, 10 μm.



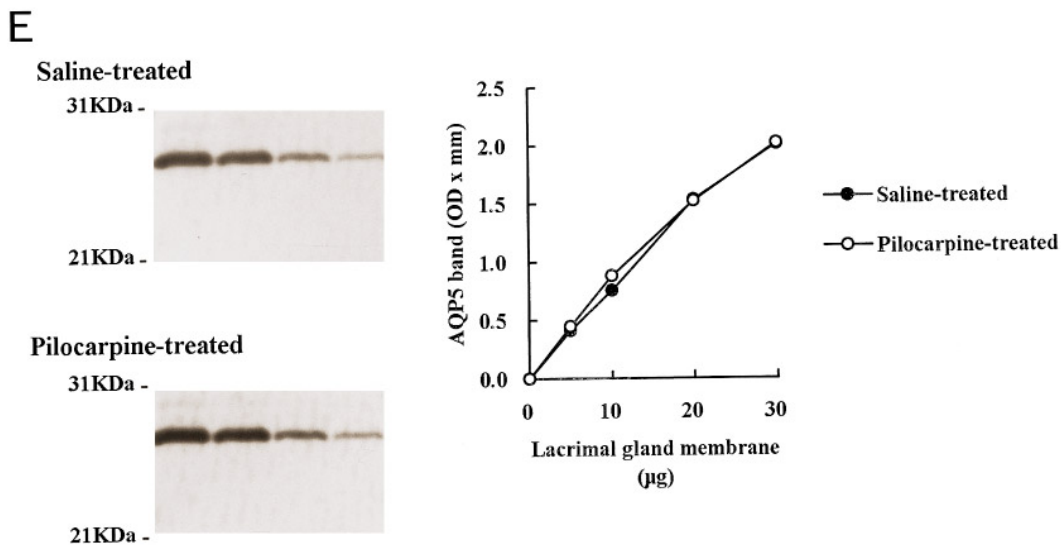


FIG. 3. Immunoblot analysis and immunoperoxidase labeling of the AQP5 in a lacrimal gland isolated from a saline-treated mouse (A and C) and a pilocarpine-treated mouse (B and D). Anti-AQP5 antibodies were raised against the carboxy terminus (A and B) and the predicted extracellular domain (C and D). (E) Western blot analysis was performed using 5, 10, 20, and 30 μg of apical membrane proteins isolated from the lacrimal glands of the treated animals. The purified antibody to AQP5 carboxy terminus was used. Densitometric analysis was performed as described in Materials and Methods. Bars: 10 μm.

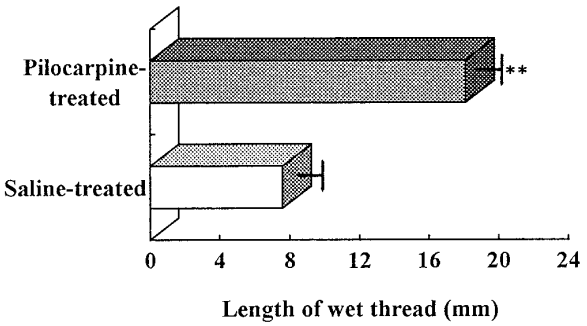


FIG. 2. Effect of pilocarpine on tear secretion in mice. Tear secretion volume is determined by the Schirmer test using a cotton thread for 5 min after systemic treatment with pilocarpine (0.05mg/10g body weight) or saline (0.05ml/10g body weight). Each value represents the mean ± SEM. **Significance ($P<0.01$) vs. saline-treated controls by paired *t*-test.

MATERIALS AND METHODS

Preparation of anti-aquaporin antibodies. Oligopeptides were synthesized using the standard solid-phase technique and purified by HPLC. Peptides were synthesized for rat AQP5 [amino acids 241-256, GTYEPEEDWEDHREER (corresponding to the C-terminus); amino acids 183-197, SMNPARSFGPAVVMN (corresponding to the extracellular domain)] and rat AQP4 (amino acids 287-301, EKGKDSSGEVLSSV, Ref. 8). AQP5 C-terminus antibodies were raised in rabbits, and other AQP5 antibodies and AQP4 antibodies were raised in guinea pigs. Antibodies were purified by affinity chromatography using a column prepared by attachment of specific peptides.

Immunohistochemistry. Five minutes after subcutaneous treatment with saline (0.05ml/10g body weight) or pilocarpine (0.05mg/10g body weight), BALB/c mice exorbital lacrimal glands were removed and fixed overnight at 4°C in Zamboni's solution or PBS containing 4% paraformaldehyde. Tissues were then cryoprotected for 6 hours with PBS containing 30% sucrose, embedded in OTC compound, and frozen in acetone/dry ice. Cryostat sections (5μm) were mounted on silanized slides (DAKO). After incubation with 0.3% H_2O_2 containing methanol, the slides were incubated for 10 minutes with PBS containing 1% BSA and then incubated with AQP5 or AQP4 antibodies (0.5-1.0 μg/ml) overnight at 4°C in PBS containing 1% BSA. The slides were rinsed with PBS 3 times for 5 minutes. The slides were incubated for 1 hour with biotinylated anti-guinea pig or rabbit IgG and rinsed with PBS. For immunofluorescence detection, slides were incubated for 1 hour with avidin-conjugated FITC (for AQP5 label) or rhodamin (for AQP4 label), washed in PBS, and mounted by using an antifade solution to retard fluorescence photobleaching. For immunoperoxidase detection, the antibody-incubated slides were incubated for 1 hour with horseradish peroxidase-conjugated avidin-biotin complex. Peroxidase activity was visualized by reaction with diaminobenzidine for 2 minutes. Slides were counterstained with hematoxylin and were photographed on Fuji Super HG-100 or HG-400 film using an Olympus fluorescence microscope or by a digital image-processing system using a BioRad MRC1024-UV confocal microscope.

Membrane preparation and immunoblot analysis. After treatment with saline or pilocarpine, the mice were sacrificed by decapitation and their exorbital lacrimal glands were removed. A membrane fraction was prepared as follows (9). The isolated lacrimal glands were rinsed with cooled saline and then were homogenized in 30mM mannitol, 10mM $CaCl_2$, 10 mM Tris-HCl, pH7.4, containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3,000*g* for 15 minutes, and the supernatant was centrifuged at 42,000*g* for 20 minutes. The resulting pellet was suspended with 100 mM mannitol, 10 mM Tris-HCl, pH7.4 and recentrifuged at 42,000*g* for 20 minutes, then the final pellet was resuspended with this buffer. The sample (apical membranes fraction) was added to a loading buffer (125mM Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol), and electrophoresed on a 10-20% polyacrylamide gradient gel. Proteins were transferred to a polyvinylidene difluoride membrane and immunolabeled using the affinity purified antibodies to AQP at a 1:1000 dilution. The bound antibodies were detected by using the ECL chemiluminescence method (Amersham). The resulting films were scanned using a laser densitometer, and the relative intensity of the AQP band peak was determined as OD × mm.

Determination of tear secretion. Tear secretion volume was determined by the Schirmer test using a Zone-Quick cotton thread treated with phenol red pH indicator (Showa Yakuhin Kako, Japan). The thread was placed on the lower eyelid of the mice for 5 minutes after their treatment with saline or pilocarpine, and tear secretion was measured as the length of the red portion of the thread in millimeters.

RESULTS AND DISCUSSION

All antibodies were affinity purified, and their specificity was determined by western blot analysis. There was no cross-reactivity between AQP5 and AQP4 antibodies, but these antibodies crossreacted between rat and mouse species on immunoblot analysis (data not shown). Figure 1 shows the localization of the AQP5 and AQP4 proteins in the mouse lacrimal glands. Immunofluorescence of the AQP5 antibody against the extracellular domain showed strong staining in the lacrimal gland cells (Fig. 1A-C). By confocal microscopy at high magnification, the fluorescein-labeled antibody was localized in the apical membrane of the acinus and duct cells. AQP4 was also expressed in the mouse lacrimal glands (Fig. 1D). A high magnification view (Fig. 1E) showed AQP4 expression on the basolateral membrane of the glandular cells. These results indicate that AQP5 and AQP4 were separately localized on the apical and basolateral sides, respectively.

Lacrimation is increased by parasympathetic nerve stimulation such as treatment with a muscarinic agonist. To confirm this phenomenon, we examined the effect of pilocarpine on tear secretion in normal mice. Figure 2 shows the measured amounts of tear secretion using the Schirmer test. The amount was determined for a 5-minute period after pilocarpine or saline treatment. The tear secretion in pilocarpine-treated mice was significantly (2.5-fold) greater than that of the saline-treated controls. We then examined whether the pilocarpine-induced increase in tear secretion is due to translocation of the AQP water channels into the plasma membrane. After the tear secretion assay, the exorbital lacrimal glands were immediately removed and fixed for immunohistological examination. Figure 3 shows the AQP5 expression in the lacrimal gland using immunoperoxidase localization and the results of western blot analysis. We used antibodies to two

regions of AQP5. The regions comprised the putative extracellular domain (loop E) and the intracellular domain (carboxy terminus) as defined by Raina et al (5). The antibody to the extracellular domain loop E showed high immunoreactivity on the apical membrane in acinar cells (Fig. 3C and D). The expression of AQP5 in the pilocarpine-stimulated mice was not significantly different from that of the saline-treated mice. In contrast, the antibody to the carboxy terminus showed very weak immunoreactivity on the apical membrane in the saline-treated mice compared with that of the pilocarpine-stimulated mice (Fig. 3A and B). Thus, the carboxy terminus-recognized anti-AQP5 antibody revealed different immunoreactivity between the pilocarpine-stimulated lacrimal glands and the saline-treated controls. The immunoreactivity of AQP4 on the basolateral membrane in the lacrimal gland was not affected by pilocarpine stimulation (data not shown).

To determine whether immunohistochemical modification results in the translocation of the AQP5 protein or new synthesis of the protein, we performed western blot analysis of the apical membrane fractions using an antibody to the carboxy terminus. Figure 3E shows the result of the immunoblot analysis. The apical membrane fractions showed a single region of immunoreactivity at 27 kDa, which was identified as the non-glycosylated AQP5 monomer (10). Relative quantification of the AQP5 protein expression was determined by densitometry of the autoradiograms. As shown in Fig. 3E, the detection of AQP5 in the saline-treated mice lacrimal glands was linear from 5 to 30 μg of apical membrane protein. Interestingly, the expression levels of AQP5 on the apical membrane in the pilocarpine-stimulated mice were not significantly different compared with the saline-treated controls. These results suggest that the change in the immunohistological response to the carboxy terminus-recognized antibody caused by pilocarpine stimulation was not related to the translocation of the AQP5.

In the present study, AQP4 and AQP5 water channels were detected in lacrimal glands, and these localizations were completely separate on the basolateral and apical membranes in the glandular cells, respectively. Water transport, tear secretion in the lacrimal glands may be mediated by both basolateral and apical aquaporins. In renal collecting duct, AQP2 and AQP3 are found in the apical and basal membranes, respectively (11). It has been demonstrated that AQP2 is predominantly a vasopressin-regulated water channel (12), and stimulation of the vasopressin induces increases in water permeability and the immunolabeling density of the AQP2 in the apical membrane (7). Based on its predicted amino acid sequence, AQP5 is more closely related to AQP2 than to the other aquaporin families. Furthermore, AQP5 contains a cAMP-dependent protein kinase consensus site, similar to the consensus site in AQP2. Because trafficking of AQP2 into the apical membrane is stimulated by vasopressin, AQP5 may be regulated by the parasympathetic nervous system, which controls tear secretion. In this study, western blot analysis using apical membrane fractions isolated from pilocarpine-stimulated lacrimal glands resulted in unchanged expression levels of AQP5 protein compared with the controls. In addition, the antibody to the AQP5 extracellular domain showed a similar immunolabeling pattern in the apical membrane in both the pilocarpine- and saline-treated lacrimal glands. We believe that stimulation of pilocarpine may produce rapid change in the gating of the AQP5 water channels but not trafficking of the channel to apical membranes. The antibody to the carboxy terminus region revealed different immunoreactivity, suggesting that this region of the protein is masked or altered in structure. We speculate that unknown regulatory proteins may associate on the carboxy terminus of the AQP5, and the proteins may be dissociated if rapid water transport is needed. Further functional studies are necessary to determine the mechanism of regulation of lacrimal gland water channel. In conclusion, AQP4 and AQP5 water channels were present in mouse lacrimal gland cells, with greater expression of the AQP4 on the basolateral membrane and of the AQP5 on the apical membrane. AQP5 carboxy terminus region was modified by pilocarpine stimulation, which increased tear secretion.

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