Immunolocalization of AQP-5 in rat parotid and submandibular salivary glands after stimulation or inhibition of secretion in vivo

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Aquaporin (AQP) water channels are expressed in a variety of fluid-transporting epithelia, and it is increasingly clear that they are likely to play a significant role in salivary secretion. Several rat exocrine glands have been shown to express AQP-5 at the luminal surface of the acinar cells. These include the lacrimal glands (11, 24), the subepithelial glands of the upper airways (26), and the submandibular and parotid salivary glands (10, 26). AQP-5 is highly expressed in the apical plasma membrane (29), and on the basis of the abundance of the channel it is predicted to play a significant role in saliva production. In support of this, knockout mice lacking AQP-5 show markedly depressed rates of salivary secretion (19, 21).

Rat salivary glands are innervated by both the sympathetic and parasympathetic branches of the autonomic nervous system. Stimulation of muscarinic receptors, α- and β-adrenoceptors, and histamine receptors with their respective agonists all induce some secretion of saliva (5). In general, parasympathetic stimulation produces the largest increase in salivary flow rate as a result of the activation of M3 muscarinic receptors on the acinar cells. Norepinephrine released from sympathetic nerve endings acts at both β- and α-adrenergic receptors. Activation of β-adrenoceptors induces secretion of the salivary proteins contained in zymogen granules by exocytosis, whereas activation of α-adrenoceptors induces modest fluid secretion (3).

AQP-5 is abundant in the apical domains of the serous acinar cells, secretory canaliculi, and intercalated duct cells, and absent from the mucous acinar cells and striated duct cells in the rat (6, 26). Within the serous acini, AQP-5 is present in the microvilli protruding into intercellular secretory canaliculi, as determined by immunoelectron microscopy (6). The issue of whether AQP-5 may be subject to regulated intracellular trafficking was raised by work on cultured cells and in vitro experiments using gland slices (13–15, 31). However, this hypothesis has not been formally tested in vivo, and no direct visualization of AQP-5 redistribution has been reported.
To evaluate whether AQP-5 is subject to intracellular translocation following stimulation or inhibition of salivation, we have undertaken a systematic investigation of the subcellular distribution of AQP-5 in rat parotid and submandibular glands after treatment with pilocarpine (a muscarinic agonist), atropine (a muscarinic antagonist), epinephrine (an α-adrenergic agonist), and phentolamine (an α-adrenergic antagonist). The effect of these agents on AQP-5 distribution was determined using laser confocal light microscopy and immunogold electron microscopy.

METHODS

Preparation of experimental animals. Seven groups of normal male Munich-Wistar rats (250–300 g body wt; n = 3/group) were maintained on a standard diet and then fasted overnight before the experiment. Animals were anesthetized with Mebumal (0.75 g/kg body wt) before all studies. Each animal was treated as described, and at the designated times, submandibular and parotid glands were perfusion fixed as described below. The drug dosages and exposure times were chosen according to comparable previous studies (2, 21–23).

Group 1 (control group) was injected intraperitoneally with 0.2 ml of physiological saline alone and fixed after 10 min. In groups 2 and 3, animals received a subcutaneous injection of pilocarpine hydrochloride (Sigma) in their necks at 10 mg/kg body wt. Animals were perfusion fixed 3 min after injection (group 2) or after 10 min (group 3). It should be noted that, in intact animals, significant salivation begins only 2–3 min after subcutaneous injection of submaximal doses of pilocarpine and reaches maximum after 10 min (23).

In group 4, intraperitoneal atropine was administered at 1 mg/kg body wt in physiological saline, and animals were fixed at 10 min. In groups 5 and 6, epinephrine hydrochloride (Sigma) was injected via the left femoral vein at 0.1 mg/kg body wt. Animals were fixed after 3 min (group 5) or 10 min (group 6).

In group 7, phentolamine (Regitine, 10 mg/ml) at 10 mg/kg body wt was injected via the left femoral vein, and animals were fixed after 10 min of treatment.

At the designated times, the submandibular and parotid glands of the experimental animals were fixed by transcardiac perfusion with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. In animals receiving phentolamine, blood pressure was monitored via a PE-50 catheter inserted into the left femoral artery (Sirecust 961 and Siredoc 220; Siemens, Munich, Germany). In animals with 10-min exposures to pilocarpine (group 3) and epinephrine (group 6), during stimulation saliva was aspirated from the mouth with a micropipette and collected in Eppendorf tubes. The volume of saliva was estimated gravimetrically. There was no measurable secretion of saliva in the control, atropine, or phentolamine groups or in the pilocarpine or epinephrine groups after 3 min of treatment.

Antibodies. For immunohistochemistry, immunofluorescence, and immunoelectron microscopy, previously characterized, affinity-purified polyclonal antibodies to rat AQP-5 (18, 26) were used. A previously characterized monoclonal Na⁺-K⁺-ATPase antibody (16)
was used as a basolateral marker for double-label immunofluorescence experiments.

**Immunohistochemistry for light microscopic examination.** The rat parotid and submandibular glands were removed and postfixed for 1 h in the same fixative solution used for perfusion, then dehydrated in ethanol followed by xylene, and finally embedded in paraffin. Sections were cut at 2-µm thickness on a rotary microtome (Leica, Bensheim, Germany) and then dewaxed and rehydrated.

For immunoperoxidase labeling (7), endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 30 min at room temperature. For antigen retrieval, sections were placed in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and heated in a microwave oven for 10 min. Nonspecific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl in 0.01 M PBS (pH 7.4) for 30 min, followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with primary antibody diluted in PBS containing 0.1% BSA and 0.3% Triton X-100. The sections were then rinsed three times in PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 10 min. The labeling was visualized with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (P448; DAKO, Glostrup, Denmark; diluted 1:200 in PBS containing 0.1% BSA and 0.3% Triton X-100) followed by incubation with 3,3-diaminobenzidine for 10 min. The sections were washed in PBS and counterstained for 1 min in Mayer’s hematoxylin, rinsed for 20 min under running tap water, dehydrated, and mounted with Eukitt mounting medium (O. Kindler). The microscopy was carried out using a Leica DMRE microscope.

Immunofluorescence double labeling was carried out with an FITC-conjugated goat anti-rabbit secondary antibody (Alexa 488; Molecular Probes Europe, Leiden, The Netherlands) for visualization of AQP-5 labeling and with a TRITC-conjugated goat anti-mouse

![Fig. 2. Immunogold electron microscopic localization of AQP-5 in rat salivary glands. A: extensive immunogold labeling is observed on microvilli and in apical membrane of intercalated duct cells of rat submandibular gland (arrows). B: immunogold labeling is associated with microvilli in intercellular secretory canaliculi of rat parotid acinar cells (arrows). C: section of rat submandibular gland, revealing extensive immunogold labeling of microvilli and of apical plasma membrane of an intercalated duct cell (arrows). Cells marked 1 and 2 are 2 adjacent cells; L, lumen; N, nucleus; TJ, tight junction.](http://ajpgi.physiology.org/)
secondary antibody (Alexa 546; Molecular Probes Europe) for visualization of Na\(^{+}\)-K\(^{+}\)-ATPase labeling. The sections were then rinsed in PBS and mounted with glycerol supplemented with antifading reagent (N-propyl gallate). The microscopy was carried out using a Leica laser confocal microscope (8, 17). Immunolabeling controls were performed using antibodies preadsorbed with the immunizing peptide (data not shown).

**Immunoelectron microscopy of rat salivary glands.** For immunoelectron microscopy, the frozen samples were freeze-substituted in a Reichert AFS freeze substitution unit (20, 25, 27, 33). In brief, the samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually raised from −80 to −70°C and, then rinsed in pure methanol for 24 h while the temperature was increased from −70 to −45°C. They were then infiltrated with 1:1 Lowicryl HM20/methanol, then 2:1, and finally pure Lowicryl HM20 before UV polymerization for 2 days at −45°C and 2 days at 0°C.

Immunolabeling was performed on ultrathin Lowicryl HM20 sections. Sections were pretreated with saturated solutions of NaOH in absolute ethanol (2–3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C with anti-AQP-5 diluted in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100 with 0.2% bovine milk (diluted 1:200). After rinsing, sections were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 10 nm of colloidal gold particles (GAR.EM10, 1:50; BioCell Research Laboratories, Cardiff, Wales, UK). The sections were stained with uranyl acetate and lead citrate before examination in Philips CM100 or Philips Morgagni electron microscopes.

Quantitation of the immunogold labeling was performed on electron micrographs from glands from three different animals within each group. The number of gold particles associated with the apical plasma membrane or with intracellular compartments (vesicles and granules) within single cells was determined. Quantitation of the electron micrograph results was carried out blindly. Data are given in means ± SE, unless indicated otherwise.

**RESULTS**

Subcellular localization of AQP-5 in salivary glands by light microscopy and immunogold electron microscopy. Immunoperoxidase labeling with a rat AQP-5 antibody and immunofluorescence double labeling for AQP-5 and Na\(^{+}\)-K\(^{+}\)-ATPase were performed on paraffin sections of rat parotid and submandibular glands. In the submandibular glands of untreated control animals (Fig. 1, A and B), AQP-5 labeling was present abundantly in the apical domains of both acinar cells (arrows) and intercalated duct cells (arrowheads). The staining appeared to be stronger in the intercalated ducts (inset in Fig. 1B), consistent with previous studies (24). In the rat parotid glands (Fig. 1, C and D) AQP-5 labeling was associated exclusively with the apical membrane of the

Fig. 3. Immunohistochemical analysis of AQP-5 distribution in rat salivary glands 3 min after pilocarpine administration. Immunofluorescence double labeling (AQP-5 and Na\(^{+}\)-K\(^{+}\) ATPase) of paraffin sections. The localization pattern does not differ from the control tissues (A and B). AQP-5 labeling (green) is apical, whereas red labeling of the basolateral membrane corresponds to Na\(^{+}\)-K\(^{+}\)-ATPase. Note that, in rat submandibular gland (A and C) AQP-5 labeling of the apical plasma membrane of the intercalated ducts (arrowheads) is overexposed to reveal the less abundant AQP-5 labeling of the acinar cells (arrows).
acinar cells and exhibited a punctate appearance. In contrast to the labeling pattern observed in the submandibular gland, no labeling was seen in the intercalated duct cells (ID, arrowheads in Fig. 1D) of the parotid. No AQP-5 labeling was observed in the striated ducts of either gland (e.g., SD in Fig. 1D). The double-labeling images also showed that there was no overlap in the distribution of AQP-5 and the basolaterally located Na\(^+\)-K\(^+\)-ATPase (34).

Immunoelectron microscopy was performed to determine the subcellular distribution of AQP-5. In the submandibular glands of control animals, extensive immunogold labeling was clearly associated with the apical plasma membranes of both intercalated duct cells (Fig. 2, A and C) and acinar cells (not shown). In rat parotid acinar cells (Fig. 2B), the labeling was especially associated with the apical microvilli, including those situated in the intercellular secretory canaliculi, consistent with previous observations (9). Immunoelectron microscopy also demonstrated that the punctate appearance of the AQP-5 distribution in both glands was due to the clustering of labeling around the microvilli separated by relatively unlabeled non-microvillous plasma membrane domains. Only very occasional gold particles were associated with intracellular vesicular structures in the subapical part of the cells. A quantitative assessment of immunogold labeling at the apical plasma membrane vs. the intracellular compartment was made on sections from three different animals within each group. On average, 7.2 ± 0.4% of the gold particles were intracellular and the remaining 92.8 ± 0.4% were localized to the apical plasma membrane domains in controls. Thus AQP-5 is highly abundant in the plasma membrane domains with little presence in intracellular structures.

Subcellular localization of AQP-5 in rat salivary glands after pilocarpine treatment. Stimulation with the muscarinic agonist pilocarpine dramatically increased saliva production, from being undetectable in the control animals to 1.2 ± 0.09 g/10 min (n = 3; mean ± SD).

Light microscope immunohistochemistry after 3 min of pilocarpine exposure revealed that the AQP-5 labeling was confined to the apical plasma membrane domains with little or no labeling of intracellular domains (Fig. 3). Compared with the controls, the apical labeling following pilocarpine administration became markedly more punctate in appearance after 10 min of drug exposure (Fig. 4) in both the submandibular and parotid glands. This seemed to be due to the combination of clustering of AQP-5 and dilatation of the lumen. Notably, both the punctate appearance (Fig. 4C) and the dilatation of the lumen (Fig. 4D) were more pronounced in the parotid gland than in the submandibular gland (Fig. 4, A and B). Moreover,
immunoelectron microscopy revealed that, as in the control animals, AQP-5 was almost exclusively associated with the apical plasma membrane domains, particularly the microvilli. This was seen after both 3 min (not shown) and 10 min of treatment. The level of intracellular labeling remained very sparse in both parotid and submandibular glands (Fig. 5). On average, 4.8 ± 1.4% of the gold particles were intracellular and 95.2 ± 1.4% were localized to the apical plasma membrane domains. Thus there was no indication of any significant change in the subcellular localization of AQP-5 in response to stimulation with pilocarpine.

**Effects of atropine on subcellular localization of AQP-5.** Treatment with the muscarinic antagonist atropine resulted in an almost complete arrest of saliva production, as evidenced by the visible drying of the oral mucosa. Immunohistochemistry and confocal laser microscopy demonstrated that the AQP-5 labeling remained confined to the apical plasma membrane domains of the acinar cells in the parotid and both the acinar and intercalated duct cells in the submandibular gland (Fig. 6). There was little evidence of any change in intracellular labeling in response to atropine treatment. Immunoelectron microscopy confirmed this and revealed almost exclusive labeling of the apical plasma membrane domains, including the microvilli of both acinar and intercalated duct cells (Fig. 7). On average, 4 ± 1 of the gold particles were intracellular, and 96 ± 1 were localized to the apical plasma membrane domains. Thus no major changes in the subcellular localization of AQP-5 were observed in response to atropine treatment.

**Effects of epinephrine on subcellular localization of AQP-5.** Stimulation with the α-adrenoceptor agonist epinephrine also increased saliva production, albeit to a much smaller extent.
than pilocarpine. Laser scanning confocal microscopy revealed that the AQP-5 labeling was confined to the apical plasma membrane domains regardless of the drug exposure time (3 or 10 min; Fig. 8). Immunoelectron microscopy confirmed these findings (data not shown). Thus there were no changes in the subcellular localization of AQP-5 compared with the control tissues.

**Effects of phentolamine on subcellular localization of AQP-5.** Treatment with the α-adrenoceptor antagonist phentolamine was carried out by monitoring of blood pressure. After 1 min of drug administration, the initial blood pressure of 90 mmHg had dropped, as expected, to 70–75 mmHg. It then remained at that level during the 10 min of treatment. Immunoperoxidase and laser scanning confocal microscopy showed that the AQP-5 labeling remained confined to the apical plasma membrane domains of acinar cells in the submandibular and parotid glands and in the intercalated duct cells in the submandibular gland. The distribution was again punctate in appearance, with little evidence of intracellular labeling (Fig. 9). Thus there were no changes in the subcellular localization of AQP-5 compared with the control tissues. This was confirmed by immunoelectron microscopy (data not shown).

**DISCUSSION**

Both the onset and the cessation of salivary secretion can occur very rapidly. This is normally attributed to the rapid kinetics of intracellular Ca\(^{2+}\) signaling and the virtually immediate response of Ca\(^{2+}\)-activated ion channels at the apical and basolateral membranes. However, the water channel AQP-5 also plays an important role in the generation of saliva, as evidenced by the reduction in volume and increase in osmolality of saliva in AQP-5-null mice (19, 21).

Several in vitro studies (13–15, 31) using membrane fractionation have shown evidence of the regulated trafficking of AQP-5 in cultured salivary gland cells and gland slices. This, therefore, has raised the possibility that AQP-5 translocation from intracellular vesicles to the apical plasma membrane may lead to an increase in water permeability after neuronal and hormonal stimulation. Although this is unlikely to occur rapidly enough to be the rate-defining step in the onset of secretion, direct examination of potential changes in AQP-5 distribution in the salivary glands of intact animals, after stimulation or inhibition of secretion, have not previously been investigated.

We have now investigated the immunolocalization of AQP-5 in rat parotid and submandibular glands fixed 3 or 10
min after in vivo administration of pilocarpine (a muscarinic agonist) or epinephrine (an α-adrenoceptor agonist) and 10 min after atropine (a muscarinic antagonist) or phentolamine (an α-adrenoceptor antagonist) administration. We found no indication of changes in the subcellular distribution of AQP-5 after any of these treatments. There was certainly no evidence of pilocarpine-induced AQP-5 translocation from intracellular vesicles or secretory granules to the plasma membrane in either the parotid or the submandibular gland. The density of gold particles in the intracellular and apical membrane structures remained the same, with just very sparse labeling of intracellular structures compared with the apical plasma membrane in all three conditions. By far the majority of labeling was always seen in the apical plasma membrane in controls or in animals subjected to any of the treatment regimens investigated.

Light microscopy also showed that the immunolocalization of AQP-5 at the apical membrane domains of the acinar cells became markedly more punctate 10 min after pilocarpine stimulation. This appeared to be due to clustering of AQP-5 at the microvilli combined with a concurrent dilatation of the lumen, most conspicuously in the parotid gland (Fig. 4C). Our immunoelectron microscopic findings confirmed that AQP-5 labeling is indeed mainly associated with the microvilli. These protrude into the acinar lumen and intercellular secretory canaliculi in both the parotid and submandibular glands. They are also present in the intercalated ducts of the submandibular gland, although there are also long stretches of apical membrane without AQP-5 labeling (Figs. 2A, 5C, 6B, and 7B).

Salivary secretion is associated with a massive exocytosis, in part a reflection of increased numbers of granule fusion sites in the cell surface (4). Because the luminal and canalicular area of the gland acinus is small, the high rate of secretory granule fusion must be matched by rapid retrieval of membrane. Although there is some dilatation of the acinar lumen, the increase in the apical membrane surface area during periods of enhanced secretion is clearly limited. The clustering of AQP-5 at the microvilli may therefore be related to the high incidence of exocytosis in the intervening regions of the apical membrane. Consistent with this explanation, Matsuzaki et al. (24) reported that treatment with the β-adrenergic agonist isoproterenol, which predominantly stimulates protein secretion, leads to a similar clustering of
AQP-5 labeling in rat parotid acinar cells together with a marked dilatation of the lumen and secretory canaliculi.

All of these observations suggest that the fusion of secretory granules with the apical membrane and their subsequent retrieval are achieved without lateral diffusion of AQP-5 into the granule membrane. This strongly indicates that, in the face of accelerated membrane turnover during stimulated secretion, AQP-5 must be anchored in place. Our findings are consistent with a model in which regions of the membrane remain tethered at the apical surface, perhaps by association with the microvilli, and other regions are targeted for secretory vesicle insertion and membrane retrieval. Thus the anchoring mechanisms that exist to retain AQP-5 in the apical membrane could account for the apparent clumping of AQP-5 labeling following stimulation with pilocarpine. Additionally, previous biochemical studies (13, 15) demonstrated partitioning of AQP-5 into an apical membrane fraction following administration of secretagogues; however, no direct visualization of AQP-5 distribution was reported. We believe that these findings may reflect a cytoskeletal reorganization associated with the retention of AQP-5 in the microvilli, because neither the immunohistochemical nor the immunoelectron microscopic studies reported here revealed an actual change in distribution.

In summary, our studies indicate that, even under basal conditions, AQP-5 is localized almost exclusively at the apical plasma membrane, particularly within the microvillous domains of the acinar cells (and the intercalated duct cells in the submandibular gland). In vivo muscarinic cholinergic and α-adrenergic stimulation and inhibition of basal secretion with muscarinic or α-adrenoceptor antagonists did not result in any significant translocation of AQP-5 between intracellular vesicles or secretory granules and the apical plasma membrane after 3 or 10 min, respectively. Even so, we cannot exclude the possibility that shorter- or longer-term stimulation or inhibition of salivary secretion might lead to a redistribution of AQP-5 between these compartments. The mechanisms regulating the anchoring of AQP-5 to apical membrane microvilli remain undefined but are likely to be a critical element of membrane dynamics during periods of stimulated secretion.

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GRANTS

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weaker labeling of the acinar cells. The immunofluorescence double labeling (AQP-5 and Na\(^+\)-K\(^+\)-ATPase) of parafﬁn sections. Localization pattern does not differ from that of control tissues (A and B). AQP-5 labeling (green) is apical, whereas red labeling of the basolateral membrane corresponds to Na\(^+\)-K\(^+\)-ATPase. Note that, in rat submandibular gland (A and C), the conspicuous difference in abundance of AQP-5 in the acini (arrows) and in the intercalated ducts (arrowheads) can be seen again. Labeling of the apical plasma membrane of the intercalated ducts is deliberately overexposed to reveal the weaker labeling of the acinar cells.

Fig. 9. Immunohistochemical analysis of AQP-5 distribution in rat salivary glands after phenolamine administration. Immunofluorescence double labeling (AQP-5 and Na\(^+\)-K\(^+\)-ATPase) of parafﬁn sections. Localization pattern does not differ from that of control tissues (A and B). AQP-5 labeling (green) is apical, whereas red labeling of the basolateral membrane corresponds to Na\(^+\)-K\(^+\)-ATPase. Note that, in rat submandibular gland (A and C), the conspicuous difference in abundance of AQP-5 in the acini (arrows) and in the intercalated ducts (arrowheads) can be seen again. Labeling of the apical plasma membrane of the intercalated ducts is deliberately overexposed to reveal the weaker labeling of the acinar cells.

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IMMUNOLOCALIZATION OF AQP-5