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Involvement of Aquaporin-5 Water Channel in Osmoregulation in Parotid Secretory Granules

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Abstract. Aquaporins (AQPs) are a family of channel proteins that allow water or very small solutes to pass, functioning in tissues where the rapid and regulated transport of fluid is necessary, such as the kidney, lung, and salivary glands. Aquaporin-5 (AQP5) has been demonstrated to localize on the luminal surface of the acinar cells of the salivary glands. In this paper, we investigated the expression and function of AQP5 in the secretory granules of the rat parotid gland. AQP5 was detected in the secretory granule membranes by immunoblot analysis. The immunoelectron microscopy experiments confirmed that AQP5 was to be found in the secretory granule membrane. Anti-AQP5 antibody evoked lysis of the secretory granules but anti-aquaporin-1 antibody did not and AQP1 was not detected in the secretory granule membranes by immunoblot analysis. When chloride ions were removed from the solution prepared for suspending secretory granules, the granule lysis induced by anti-AQP5 antibody was inhibited. Furthermore, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, an anion channel blocker, blocked the anti-AQP5 antibody-induced secretory granule lysis. These results suggest that AQP5 is, expressed in the parotid gland secretory granule membrane and is involved in osmoregulation in the secretory granules.

Key words: Aquaporin-5 — Secretory granules — Osmoregulation — Cl^- Conductance — Parotid gland

Introduction

Aquaporins (AQPs) are a family of small integral membrane proteins with six hydrophobic, α-helical, membrane-spanning domains surrounding a highly selective aqueous pore. They function as channels that permit water or very small solutes to pass, and have been identified in the plasma membranes of many fluid-transporting epithelia and endothelia (Borgnia et al., 1999; Verkman & Mitra, 2000). At least 11 AQPs have been identified in mammals, each with a distinctive tissue distribution pattern. Of these, AQP1, AQP4, AQP5 and AQP8 have so far been identified in mammalian salivary glands. AQP1 is expressed mainly in the capillary endothelial cells of rat parotid and submandibular glands (Nielsen et al., 1993; Li et al., 1994). AQP4 has been detected in the excretory ducts of rat salivary glands (Frigeri et al., 1995). Evidence for the expression of AQP8 in the submandibular glands of rats and mice (Koyama et al., 1997; Ma, Yang & Verkman, 1997) has been reported, although the localization of AQP8 has yet to be established. AQP5, which was originally cloned from the rat submandiular gland (Raina et al., 1995), is localized in the apical membrane of the serous acinar cells of rat submandibular and parotid glands (He et al., 1997; Funaki et al., 1998). In human salivary glands, localization of AQP5 in the luminal and canalicular membranes of serous and mucous acinar cells has been demonstrated (Gresz et al., 2001). Although its function is not completely resolved, AQP5 is believed to provide the main pathway for osmotic water flow from the acinar cells to the lumen, because mice lacking AQP5 show depressed salivary secretion, and the small amount of saliva that is secreted is markedly hypertonic (Ma et al., 1999; Krane et al., 2001).

Recently, AQPs have been reported to be present in intracellular organelles. Localization of AQP6 in intracellular vesicles has been demonstrated in renal epithelium (Yasui et al., 1999). In human parotid glands, AQP5 has been reported to localize in the cytoplasmic vesicles of the acinar cells (Smith et al.,

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1999). In rat exocrine pancreas, AQP1 has been demonstrated to be localized in zymogen granules and to be involved in GTP-mediated water entry and swelling (Cho et al., 2002). In parotid acinar cells, secretion of proteins, such as amylase and antimicrobial and remineralizing proteins, is a primary function. Since the protein is concentrated and condensed in the secretory granules, regulation of intragranular osmolarity is important, and it is possible that permeation of ions and water contributes to the osmoregulation. The presence of Cl^- channels has been demonstrated (Gasser & Hopfer, 1990) in these granules, but no AQPs were detected as yet in salivary secretory membranes. In this paper, we demonstrate that AQP5 is localized in the secretory granules of the rat parotid gland and is involved in granule osmoregulation.

Materials and Methods

Materials

Anti-AQP polyclonal antibodies were purchased from Chemicon (CA). Alexa Fluor 488 goat anti-rabbit IgG for immunofluorescence was purchased from Molecular Probes (OR). Percoll and ECL were purchased from Roche (Switzerland). Block Ace was purchased from Yukijirushi-Nyugyo (Japan). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was purchased from Biomol (PA). O.C.T. compound (Tissue-Tek) was purchased from Sakura Fine Technical Co. (Japan). MAS-coated slides for mounting frozen sections were purchased from Matsunami Glass Ltd. (Japan). LR white resin and EPON-812 were purchased from London Resin, (England) and TAAB (England), respectively.

Preparation of Secretory Granules and Granule Membranes

Parotid glands were isolated from sodium pentobarbital-anesthetized (50 mg/kg) male Sprague-Dawley rats (180–200 g). Secretory granules of the rat parotid glands were isolated by Percoll-gradient centrifugation as previously described (Dohke et al., 1998; Fujita-Yoshigaki et al., 1999). To prepare granule membranes, the isolated granules were treated with low osmotic Buffer A (2 mM MOPS, pH 6.8, 0.4 mM PMSF) and centrifuged at $100{,}000 \times g$ for 60 minutes. The resulting pellet was suspended in Buffer A and used as the granule membrane fraction. Secretory granules of rat pancreas were prepared by the method previously described (Nadin et al., 1989).

Preparation of Membrane Fraction of Parotid Gland

Parotid gland membranes were prepared by Percoll gradient centrifugation as previously described (Takuma & Baum, 1985). The isolated membrane fraction containing apical membranes was suspended with 10 mM Tris-HCl buffer (pH 7.6) containing 0.25 M sucrose and 0.1 mM PMSF.

Western Blotting

Proteins were resolved by 15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (12.5 V, overnight). The membrane was blocked at room temperature for 60 min in Block Ace, and probed for 120 min with the first antibody. The blots were washed three times with 10% Block Ace containing 0.05% Tween 20 and probed for 90 min with the second antibody. Immunoreactivity was determined by means of the ECL chemiluminescence reaction.

Conventional Immunofluorescence Microscopy

Fresh parotid glands isolated from anesthetized rats were immediately embedded in O.C.T. compound and rapidly frozen by a metal block cooled with liquid nitrogen. Frozen sections about 5 μm thick were prepared by slicing the samples at −20 °C, and were mounted onto MAS-coated glass slides. After fixation with 100% methanol and rinsing with PBS, these sections were incubated with anti-AQP5 polyclonal antibody (1:50) at 4 °C for 2 hours. After being rinsed twice with PBS, the specimens were incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG at 4 °C for 1 h. Sections were examined and photographed with a conventional fluorescence microscope (Axiophoto 2: Carl Zeiss Co., Germany).

Immunoelectron Microscopy

For ultra-thin cryosection, parotid glands isolated from anesthetized rats were fixed by perfusion with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 0.5 h at 4 °C. Small pieces were immersed in 2.3 M sucrose in PBS (pH 7.2) for 1 h, mounted on specimen holders, and quickly frozen in liquid nitrogen. Silver-to-gold ultra-thin frozen sections were cut at −100 °C on a microtome equipped with a FC4-S cryochamber (Reichert-Nissei, Japan). The sections were transferred onto a drop of 2.3 M sucrose, and were mounted with Formvar-coated nickel grids. Then they were processed for immunostaining. The samples floated on a drop of the anti-AQP5 polyclonal antibody (1:100) were incubated at 37 °C for 2 h and rinsed with PBS. Subsequently, the sections were transferred onto a drop of 10-nm colloidal gold conjugated with anti-rabbit IgG (1:50) and incubated at 37 °C for 1 h. The bound antibodies were fixed with 1% glutaraldehyde in 120 mM phosphate buffer (pH 7.2) for 10 minutes, osmicated, dehydrated, and then embedded in LR white resin.

Isolated intact secretory granules were immunoreacted directly with anti-AQP5 antibody (1:50) in Buffer B (250 mM sucrose, 0.1 mM PMSF, 5 mM MOPS, pH 6.8) at room temperature for 30 minutes. For washing, the secretory granules were suspended with Buffer B and centrifuged twice at $600 \times g$ for 10 min. The specimens were resuspended in Buffer B containing 5-nm colloidal gold-conjugated anti-rabbit IgG (1:50) and incubated at room temperature for 15 min. The specimens were then washed twice and fixed with fixative solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After fixation, the specimens were immersed in 3% low-melting-point agarose in 0.1 M phosphate buffer (pH 7.2) and cooled. The agarose containing secretory granules was cut into 1 mm squares. The agarose blocks containing secretory granules were fixed with fixative solution at 4 °C for 10 min. After osmication at room temperature for 30 min in 1% osmium tetraoxide, these samples were dehydrated in a graded series of ethanol and propylene oxide and then embedded in EPON-812.

These ultra-thin cryosections and conventional ultra-thin sections were stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (H-7100: Hitachi, Co., Tokyo).

Measurement of Granule Osmotic Lysis

Secretory granules were suspended in 1 ml buffered iso-osmotic KCl solutions (in mM: 150 KCl, 1 EGTA, 0.1 $MgSO_4$, and 20

HEPES, pH 7.2) and osmotic lysis was started by addition of $HgCl_2$ or anti-AQP5 antibody. The kinetics of lysis was monitored by measuring the time-dependent changes in the light scatter absorbance of the granule suspension at 540 nm as previously described (Gasser & Hopfer, 1990). Absorbance was measured continuously for 60 min at 37 °C, using a spectrophotometer (Shimadzu UV-265FW, Japan).

Particle Size Analysis by Photon Correlation Spectroscopy

Change of secretory granule size was measured by the photon correlation spectroscopy instrument N5 (Beckman Coulter, USA). Rat parotid secretory granules were suspended in 1 ml Buffer B. The 10 μl granule fraction was suspended in 1 ml iso-osmotic KCl solution and transferred to a plastic cuvette at room temperature. The cuvette was set in N5 and focused by 25 mV Helium-Neon laser, and the scattering light at angle 90 ° was detected at 20 min.

Protein Assay

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Statistical Analysis

Significant differences were determined using Student's *t*-test. A P value < 0.05 was considered to be statistically significant.

Results

AQP5 in Secretory Granule Membranes

The secretory granules of the rat parotid gland were isolated (Fig. 1*A*), and the localization of AQPs in the granule membranes was determined. Anti-AQP5 antibody specifically recognized a 27-kDa band on immunoblots of the secretory granule membranes (Fig. 1*B*), but anti-AQP1, anti-AQP4 or anti-AQP8 antibodies did not (*data not shown*). The same size of an immunoreactive band was detected in the membrane fraction isolated from the rat parotid gland (Fig. 1*B*). These bands were not detected when the blot was reacted with the anti-AQP5 antibody preabsorbed with a molar excess of the AQP5 COOH-terminal peptide used for the immunization (*data not shown*). These results indicate that AQP5 localizes in the secretory granule membranes of the rat parotid gland.

To confirm the localization of AQP5 in the secretory granule membranes, morphological studies were carried out. In conventional Immunofluorescence microscopy, AQP5 fluorescence was not only clearly detected in the apical membranes of the parotid acinar cells, as previously described (Nielsen et al., 1997), but was also diffusely spread through other zones of the cytoplasm (Fig. 2*A*). This observation suggests that AQP5 localizes in the secretory granules. Thus, the localization was determined with

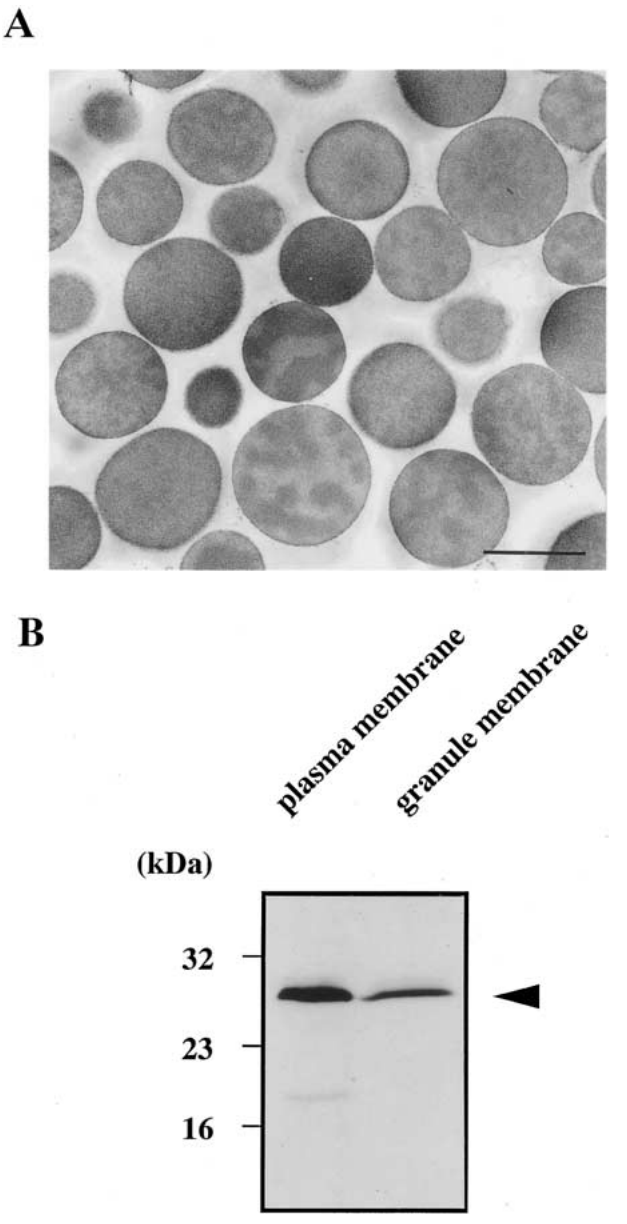

Fig. 1. Immunoblot analysis of localization of AQP5 in rat parotid secretory granules. (*A*) Secretory granules from the rat parotid gland were isolated as described under Materials and Methods. Bar, 1 μm. (*B*) Rat parotid secretory granule membrane and plasma membrane fractions (50 μg) were fractionated electrophoretically on a 15% SDS-polyacrylamide electrophoresis gel, blotted, then probed with rabbit polyclonal anti-AQP5 antibody. The results are representative of 5 independent experiments.

cryostat sections by immunoelectron microscopy. In transmission electron micrographs, gold particles indicating positive reactions for AQP5 were found at the apical membranes of acinar cells (Fig. 2*B* and *C*) and the secretory granule membranes (Fig. 2*B*). When the secretory granules isolated were used for immunoelectron microscopy with anti-AQP5 antibody, the gold particles were clearly ordered in a

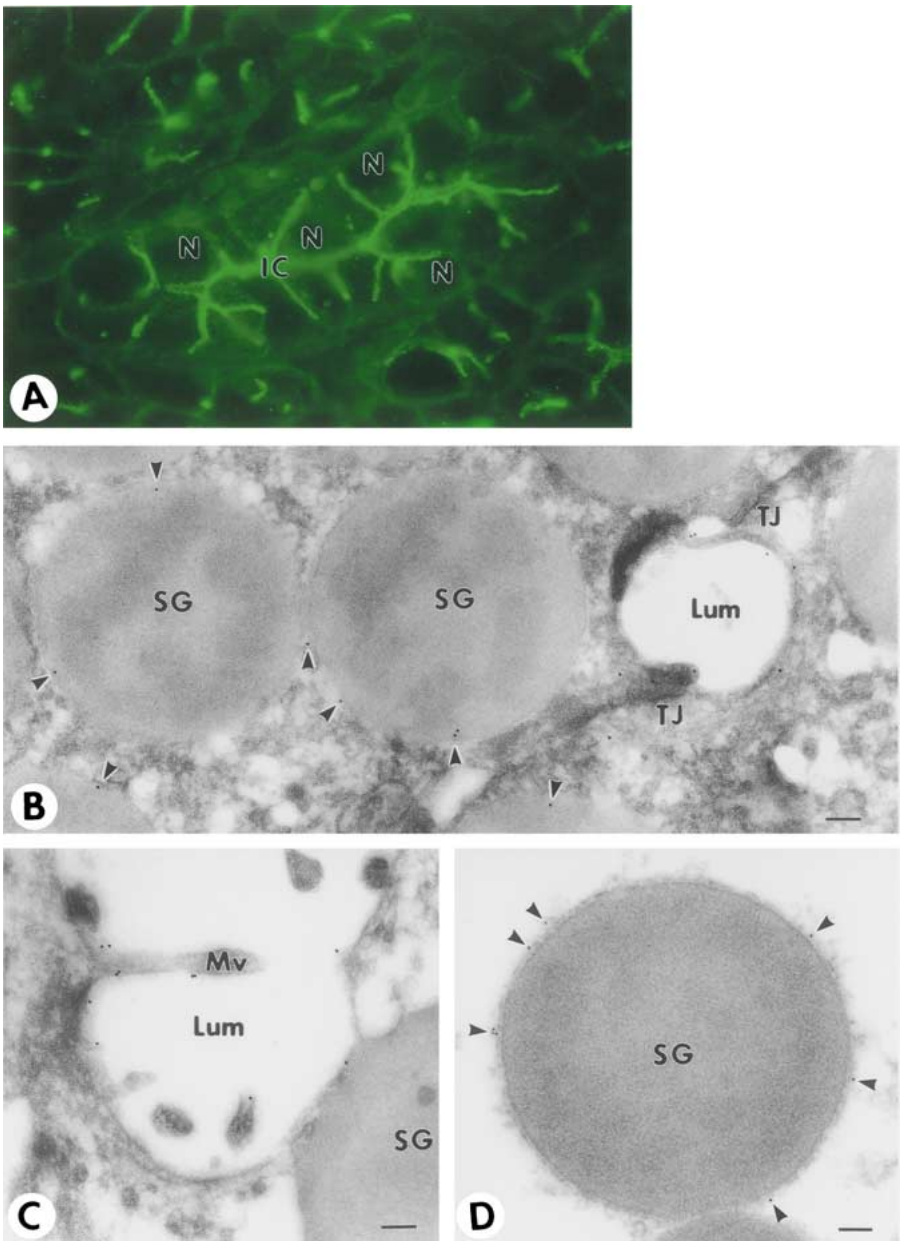

Fig. 2. Localization of AQP5 in secretory granules of the rat parotid gland. (*A*) Conventional immunofluorescence microscopy. AQP5 fluorescence was seen not only clearly in the apical membranes but also diffusely in other zones of cytoplasm in the rat parotid gland (×900). *N*, nuclei; *IC*, intracellular canaliculi. (*B*) and (*C*) AQP5 labeling of secretory granule membrane (*arrowheads*) in ultra-thin cryosection. AQP5 labeling can be seen in luminal membranes (*C*) and secretory granule membranes (*B*, *arrowheads*). SG, secretory granules; *Lum*, lumen; *TJ*, tight junction; *Mv*, microvilli; Bar, 0.1 μm. (*D*) AQP5 labelings (*arrowheads*) in an intact secretory granule. Secretory granules were incubated with the anti-AQP5 antibody and 10 nm gold particles as described under Materials and Methods. Bar, 0.1 μm.

single line at the secretory granule membrane (Fig. 2*D*). These observations confirmed that AQP5 is localized in the secretory granule membranes of rat parotid acinar cells.

Anti-AQP5 Antibody-induced Secretory Granule Lysis

Because anti-AQP antibodies are useful for studies with AQP functions (Cho et al., 2002; Zhu et al., 2002), we next examined the effect of anti-AQP5 antibody on secretory granule lysis. When isolated secretory granules were suspended with iso-osmotic KCl solution and incubated, some granule lysis occurred (Fig. 3*A*). In the presence of 5 μg/ml anti-AQP5 antibodies, granule lysis was clearly facilitated (Fig. 3*A* and *B*). The anti-AQP5 antibody pretreated with a molar excess of the AQP5 COOH-terminal peptide did not induce the facilitation of the granule lysis (*data not shown*). A specific antibody against AQP1, which was not detected in parotid secretory granule membranes by immunoblotting, failed to induce osmotic granule lysis (Fig. 3*B*). Anti-AQP4, anti-APQ7 and anti-AQP9 antibodies did not induce secretory granule lysis either (*data not shown*). The anti-AQP5 antibody-induced granule lysis did not occur in zymogen granules isolated from rat pancreas (*data not shown*). These results suggest that binding antibody to AQP5 in these granules results in lysis of the secretory granules from rat parotid gland.

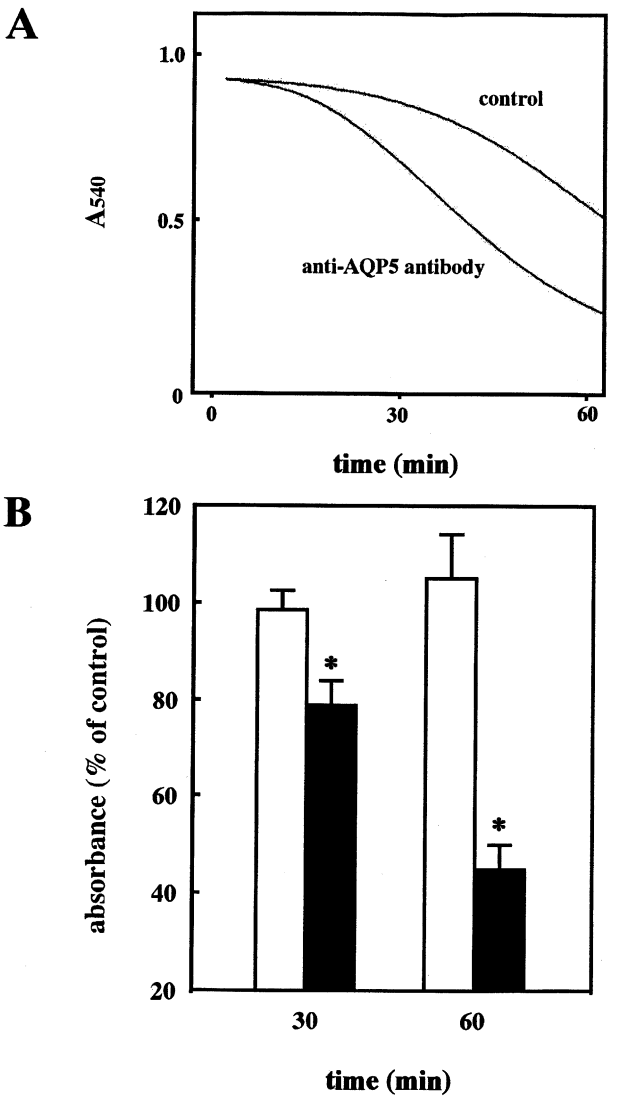

Fig. 3. Secretory granule lysis induced by Anti-AQP5 antibody. (*A*) Parotid secretory granules were suspended in isotonic KCl solution and incubated at 37 °C for 1 h without or with anti-AQP5 antibody (5 μg/ml). (*B*) Secretory granules were incubated with anti-AQP1 (*white columns*) or anti-AQP5 antibodies (*black columns*) for 30 and 60 min. Results are means ± SEM of 3 independent experiments. $*P < 0.05$.

Since it has been known that swelling results in secretory granule lysis (Gasser & Hopfer, 1990), we determined, using a dynamic light scattering system (the N5 Submicron Particulate Size Analyzer, Beckman Coulter), whether anti-AQP5 antibody induces swelling of secretory granules. The peak value of whole granule size was approximately 1,200 nm in the absence of the antibody (Fig. 4, *dashed line*). On the other hand, the peak value increased to approximately 1,900 nm by the addition of anti-AQP5 antibody (Fig. 4, *solid line*), indicating that the number of swelled secretory granules has been increasing in the presence of anti-AQP5 antibody. Therefore, it is conceivable that the secretory granule lysis follows granule swelling.

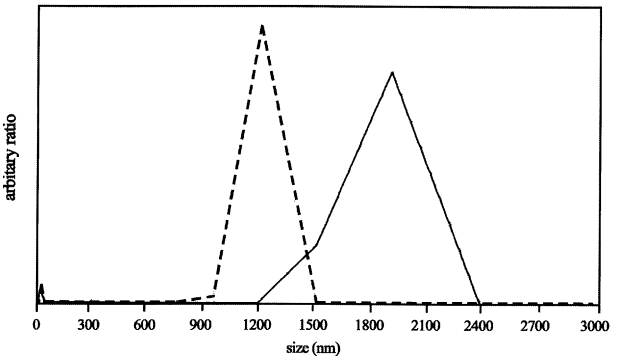

Fig. 4. Swelling of secretory granules induced by anti-AQP5 antibody. Secretory granules were suspended with isotonic KCl solution and incubated without (*dashed line*) or with (*solid line*) 5 μg/ml anti-AQP5 antibody for 20 min at room temperature. Scattered light from 25 mV Helium-Neon laser focused solution was detected. Results are representative of 8 independent experiments.

Effect of Cl^- Transport into Secretory Granules on Granule Lysis Induced by Anti-AQP5 Antibody

In the secretory granules of the rat parotid gland, a Cl^- influx pathway, followed by water movement, has been demonstrated (Smith et al., 1999). Therefore, we investigated the relationship between the secretory granule lysis induced by anti-AQP5 antibody and Cl^- transport in rat parotid secretory granules. When parotid secretory granules were suspended in iso-osmotic mannitol or K-gluconate solutions instead of KCl solution, anti-AQP5 antibody-induced granule lysis was completely blocked (Fig. 5*A*). Anion channel blockers have been reported to decrease Cl^- conductance in rat parotid secretory granules (Smith et al., 1999). Therefore, we examined the effect of DIDS, an anion channel blocker, on the secretory granule lysis induced by anti-AQP5 antibody. In the presence of 100 μM DIDS, the secretory granule lysis induced by anti-AQP5 antibody was completely blocked (Fig. 5*B*). These results suggest that Cl^- conductance is closely related to AQP5 function in the secretory granules of the parotid gland.

Discussion

It has been known that AQP5 localizes in the luminal membrane of the acinar cells of rat and human salivary glands (He et al., 1997; Funaki et al., 1998; Gresz et al., 2001). In this paper, we demonstrated by immunoblotting and immunohistochemical studies that AQP5 was expressed in the secretory granule membranes in the rat parotid gland.

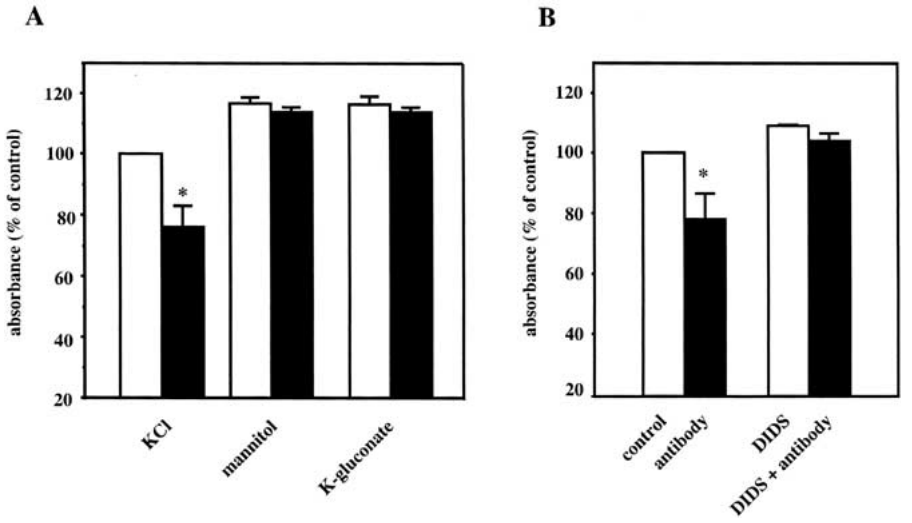

Fig. 5. Inhibition of anti-AQP5 antibody-induced secretory granule lysis in Cl^- free solution and in the presence of DIDS. (*A*) Parotid secretory granules were suspended in isotonic KCl, mannitol or K-gluconate solutions and incubated at 37 °C for 30 min without (*white columns*) or with (*black columns*) anti-AQP5 antibody (5 μg/ml). (*B*) Secretory granules were suspended in isotonic KCl solution and incubated with anti-AQP5 antibody (5 μg/ml) at 37 °C for 30 minutes in the absence or presence of 100 μM DIDS. The lysis of secretory granules in isotonic KCl solution without anti-AQP5 antibody was indicated as 100% (*control*). Results are means ± SEM of 3 independent experiments. $*P < 0.05$.

In the rat pancreatic zymogen granules, the presence of AQP1 has been reported (Cho et al., 2002). Since the protein band and gold particles immunoreacted to the antibody raised to the carboxyl terminus of AQP1 were detected in the zymogen granules permeabilized with streptolysin-O, but not in the intact granules using immnunoblotting and immunoelectron microscopy, the carboxyl domain of AQP1 has been suggested to lie on the inner side of the granule membrane. In contrast, as shown here, gold particles immunoreacted with the antibody raised to the carboxyl terminus of AQP5 were clearly ordered in a single line at the membrane of the intact secretory granule isolated from the rat parotid gland. This observation indicates strongly that the carboxyl domain of AQP5 is situated on the extragranular side.

We demonstrated here that the antibody against AQP5 induced lysis of secretory granules from rat parotid gland. In cells, changes of cell volume, shrinkage and swelling are thought to occur as a result of an imbalance between the influx and efflux of ions. The resulting change in tonicity requires a rapid and regulated change in cell water permeability. Membrane proteins, such as water channels, ion channels and cotransporters, and simple permeability of membrane to water have been considered to contribute to the water permeability (Zeuthen & MacAulay, 2002; Hill, Shachar-Hill & Shachar-Hill, 2004). Since secretory proteins are concentrated and stored in secretory granules, it is conceivable that such multi-mechanisms involved in water permeability contribute to intra-granular osmoregulation. Therefore, it is unlikely that anti-AQP5 antibody completely inhibits secretory granule water permeability. It is rather possible that the secretory granule lysis is induced by an imbalance between water and ion channel functions in the secretory granules. The secretory granule membranes in the rat parotid gland have been demonstrated to have Cl^- conductance, which regulates Cl^- transport (Gasser & Hopfer, 1990; Gasser, Goldsmith & Hopfer, 1990). In this paper, we also showed that the anti-AQP5 antibody-induced secretory granule lysis is completely inhibited when the secretory granules are suspended with iso-osmotic mannitol solution or potassium-gluconate solution instead of potassium chloride. Furthermore, the anti-AQP5 antibody-induced secretory granule lysis was inhibited when the granules were suspended with iso-osmotic KCl solution containing DIDS, an anion channel blocker. It is widely believed that AQPs are virtually impermeable to ions (Agre et al., 1997; Saparov et al., 2001). There is no evidence that AQP5 acts as ion channels in the AQP5-expressing oocytes (Agre et al., 1997; Anthony et al., 2000), although AQP1 and AQP6 can act as ion channels (Anthony et al., 2000; Hazama et al., 2002). It is therefore unlikely that AQP5 functions as a Cl^- channel. Considering these facts, it is conceivable that a balance of water permeation via AQP5 and Cl^- conductance is necessary for secretory granule osmoregulation. Recently, the hypothesis that AQPs function as osmotic and turgor sensors rather than water channels, the sensor hypothesis, has been advocated (Shachar-Hill & Hill, 2002; Hill, Shachar-Hill & Shachar-Hill, 2004). Therefore, AQP5 appears to act as an osmosensor in the secretory granules of the parotid gland, although precise mechanisms of the relationship between AQP5 and the Cl^- channels are still obscure.

In this paper, we have demonstrated a constitutive osmoregulation of secretory granules. On the other hand, the swelling of secretory granules is considered to be a critical process for exocytosis. In the pancreatic zymogen granules, AQP1 has been reported to present in zymogen granule membranes and to regulate granule water entry and swelling mediated by GTP, a putative factor involved in exocytosis (Cho et al., 2002). In the secretory granules of the rat parotid gland, it has been considered that Cl^- influx followed by water movement is required for the exocytosis via both swelling and the flushing out of granular contents into the lumen (Gasser & Hopfer, 1990). In rat parotid acinar cells, the Cl^- conductance has been reported to be regulated by GTP-binding proteins (Watson et al., 1997). Furthermore, apical staining for AQP5 tended to appear as clusters of dots in the submembranous areas by the β-agonist isoproterenol, which can stimulate exocytosis (Matsuzaki et al., 1999). Therefore, AQP5 appears to be involved in the regulation of exocytosis.

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