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Involvement of AQP6 in the Mercury-Sensitive Osmotic Lysis of Rat Parotid Secretory Granules

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Abstract In secretory granules and vesicles, membrane transporters have been predicted to permeate water molecules, ions and/or small solutes to swell the granules and promote membrane fusion. We have previously demonstrated that aquaporin-6 (AQP6), a water channel protein, which permeates anions, is localized in rat parotid secretory granules (Matsuki-Fukushima et al., Cell Tissue Res 332:73–80, 2008). Because the localization of AQP6 in other organs is restricted to cytosolic vesicles, the native function or functions of AQP6 in vivo has not been well determined. To characterize the channel property in granule membranes, the solute permeation-induced lysis of purified secretory granules is a useful marker. To analyze the role of AQP6 in secretory granule membranes, we used Hg^{2+}, which is known to activate AQP6, and investigated the characteristics of solute permeability in rat parotid secretory granule lysis induced by Hg^{2+} (Hg lysis). The kinetics of osmotic secretory granule lysis in an isoosmotic KCl solution was monitored by the decay of optical density at 540 nm using a spectrophotometer. Osmotic secretory granule lysis was markedly facilitated in the presence of 0.5–2.0 μM Hg^{2+}, concentrations that activate AQP6. The Hg lysis was completely blocked by β-mercaptoethanol which disrupts Hg^{2+}-binding, or by removal of chloride ions from the reaction medium. An anion channel blocker, DIDS, which does not affect AQP6, discriminated between DIDS-insensitive and sensitive components in Hg lysis. These results suggest that Hg lysis is required for anion permeability through the protein transporter. Hg lysis depended on anion conductance with a sequence of $NO_3^- > Br^- > I^- > Cl^-$ and was facilitated by acidic pH. The anion selectivity for NO_3^- and the acidic pH sensitivity were similar to the channel properties of AQP6. Taken together, it is likely that AQP6 permeates halide group anions as a Hg^{2+}-sensitive anion channel in rat parotid secretory granules.

Keywords Aquaporin 6 · Parotid gland · Secretory granules · Channel · Osmoregulation · Sprague–Dawley rats

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Introduction

Thirteen mammalian isoforms of aquaporins (AQPs) have been identified as membrane proteins that function as channels for water and small solutes (Gomes et al. 2009). The presence of organelle AQPs has been found in mitochondria, the endoplasmic reticulum, and in intracellular vesicles and secretory granules (Arnaoutova et al. 2008; Calamita et al. 2007; Jeremic et al. 2005; Matsuki et al. 2005; Okada et al. 2008). One AQP family member, AQP6, was identified in cytoplasmic vesicles of acid secreting α-intercalated cells from renal collecting ducts (Yasui et al. 1999b). AQP6 is considered to function as an anion channel rather than a water channel as determined by patch clamp recordings (Yasui et al. 1999a). AQP6 conductance can be activated by Hg^{2+} or acidic pH as verified by single

channel recording in oocytes (Hazama et al. 2002). In HEK293 cells expressing a GFP-tag on the *N*-terminus of AQP6, AQP6 was localized in the plasma membrane and anion conductance was observed in response to Hg^{2+} or an acidic pH (Ikeda et al. 2002). In AQP6 expressed in oocytes and in HEK293 cells, the sequence of halide group anion permeability was $NO_3^- >> Br^- > I^- > Cl^-$ (Hazama et al. 2002; Ikeda et al. 2002). Those findings strongly support the function of AQP6 as a NO_3^- permeable anion channel in mammalian cells.

AQP6 is unique in permeating anions activated by acids or Hg^{2+}. Therefore, the endogenous role of AQP6 has been studied in various organs. The presence of AQP6 has been demonstrated not only in the kidney but also in multiple other organs (Iandiev et al. 2011; Jeremic et al. 2005; Laforenza et al. 2009; Lopez et al. 2007; Taguchi et al. 2008). In those organs, AQP6 was shown to mostly localize in the cytoplasm of cells. However, those organs did not have sufficient numbers of AQP6-expressing cells to collect and purify AQP6-containing vesicles. Therefore, the endogenous function or functions of AQP6 in vivo has not been well determined.

Ion transport of secretory (zymogen) granules and secretory vesicles has been considered to be essential for hormones, neurotransmitters and the secretion of digestive enzymes (Gasser et al. 1988, 1990; Gasser and Hopfer 1990; Thevenod et al. 1990). From those studies, secretory granule swelling has been hypothesized to be a prerequisite for secretory granule fusion with the plasma membrane during the process of exocytosis. In rat parotid secretory granules, anion transport pathways have been determined by the technique of ionophore-induced lysis in iso-osmotic salt solutions (Gasser et al. 1990; Gasser and Hopfer 1990; Gomes et al. 2009; Matsuki et al. 2005). Purified parotid secretory granules are relatively stable in physiological conditions of pH, temperature and ionic strength. The secretory granule lysis can be recorded by spectrophotometry as the decay of scattered light, and that assay technique has been used to study the transporter property in secretory granules. Such secretory granule lysis is reproducible using drugs such as valinomycin (a K^+ ionophore), CCCP (a H^+ ionophore) or nigericin (a K^+–H^+ exchanger). These types of granule lysis require anion transport coupled with water movement.

Recently, we reported that AQP6 is expressed in rat parotid acinar cells and is localized both in plasma membranes and in secretory granule membranes (Matsuki-Fukushima et al. 2008). From the channel property of AQP6, we predicted that AQP6 localizes as an anion channel in rat parotid secretory granule membranes and contributes to anion transport. In this study, we examined the physiological characteristics of Hg^{2+}-induced osmotic lysis of rat parotid secretory granules, and the results suggest the involvement of AQP6 in osmoregulation.

Materials and Methods

Preparation of Parotid Secretory Granules

Parotid secretory granules were purified from 6 week old Sprague–Dawley male rats and were purified by Percoll-gradient centrifugation as previously described (Fujita-Yoshigaki et al. 1996). Purified secretory granules were resuspended and kept in 0.3 ml homogenization buffer (300 mM sucrose, 2 mM MOPS, pH 6.8, 1 mM benzamidine, 0.4 mM PMSF) on ice.

Granule Osmotic Lysis Assay

Osmotic granule lysis was recorded as previously described (Gasser et al. 1990). Briefly, 20–30 µl of suspension of purified secretory granules were resuspended in 500 µl of iso-osmotic KCl solution (150 mM KCl, 1 mM EGTA, 0.1 mM $MgSO_4$ and 20 mM HEPES, pH 7.2). When granules were suspended in iso-osmotic KCl solution, granule suspension was cloudy as a result of the Tyndal effect. When osmotic lysis occurred, the suspension become translucent. We measured the changing of turbidity by using spectrophotometry at fixed wavelength of visible light. To examine the assay, the starting optical density (OD) of the secretory granule suspension was adjusted to 0.8–0.9 at an absorbance 540 nm. The kinetics of secretory granule lysis was monitored by measuring the time-dependent changes in the OD of the secretory granule suspension at 540 nm for 15 min continuously with Hg^{2+} (Fig. 1a) or at time points of 0 and 15 min (in other experiments) at 37 °C using a spectrophotometer (Beckman Coulter, Brea, CA). DIDS was purchased from Enzo Lifesciences (Plymouth Meeting, PA) and was added to the reaction medium 10 min before the recording. The ratio of the granule lysis is defined as the percentage of the initial value.

The DIDS-insensitive component in Hg lysis was calculated as shown in Fig. 2. First, the Hg lysis group was subtracted from the control group, yielding the "Hg^{2+} sensitive component," indicated as (a). Next, the group Hg lysis in the absence of DIDS was subtracted from the group Hg^{2+}-induced granule lysis in the presence of DIDS, and the remaining component is the "DIDS-insensitive component," indicated as (c). Finally, the DIDS-insensitive component was subtracted from the Hg^{2+}-sensitive component, indicated as (b). Calculated data are shown in the table beside the bar graph in Fig. 2b.

Statistical Analysis

Significant differences were determined using Student's *t* test (Figs. 1, 2) or repeated measures one-way ANOVA

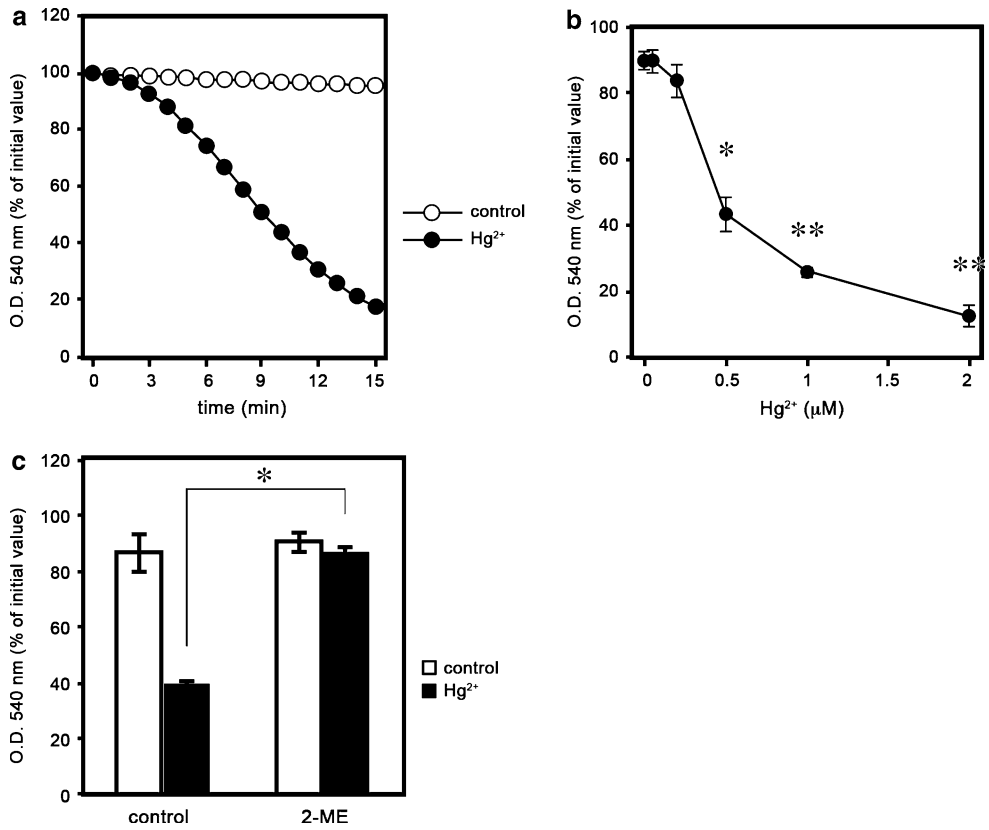


Fig. 1 Osmotic parotid granule lysis induced by Hg^{2+}. Osmotic granule lysis is reflected in the decrease of OD at 540 nm. **a** Purified rat parotid secretory granules are suspended in an iso-osmotic KCl solution, and the OD is slowly decreased (*open circles*). In the presence of Hg^{2+} (1 μM), a known activator of AQP6, the OD rapidly decreased within 20 min (*solid circles*). The trace shown represents typical direct recordings of 5 experiments obtained from different preparations of parotid secretory granules. **b** Hg^{2+}-induced granule lysis is concentration dependent. **c** Hg^{2+}-induced granule lysis is completely suppressed by 10 mM 2-ME (*solid bar*). Values are expressed as mean ± SEM ($n = 3$)

(Fig. 3). A P value of <0.05 (indicated with one asterisk) or <0.005 (indicated with two asterisks) is considered to be statistically significant.

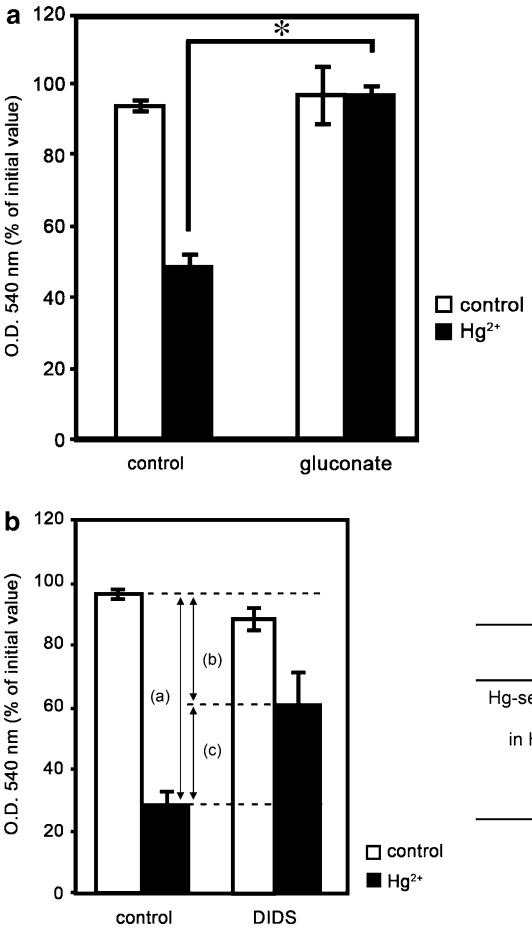
Results

Hg^{2+} Induces Lysis of Rat Parotid Secretory Granules

To determine the effects of Hg^{2+} on rat parotid secretory granules, the granule osmotic lysis assay was carried out. When purified rat parotid secretory granules were suspended in an iso-osmotic KCl solution, the OD slowly decreased. It is considered that the reduction of the OD is caused by osmotic granule swelling and lysis (Gasser et al. 1988). Upon application of 1 μM Hg^{2+}, the OD rapidly decreased to the baseline within 15 min. The Hg^{2+}-induced decay in the OD was recorded over concentrations between 0.5 and 2.0 μM. 2-Mercaptoethanol (2-ME), a protective reagent for the sulfhydryl group in cysteine residues, completely suppressed the Hg^{2+}-induced decrease of OD. These results suggest that facilitated granule lysis by Hg^{2+} is caused by the binding to cysteine residues of protein or proteins in the secretory granule membranes (Fig. 1c).

Hg^{2+}-Induced Granule Lysis (Hg Lysis) is Coupled with Chloride Permeability

Our laboratory has previously shown that the rat parotid granule membrane has a Cl^- transport pathway, followed by water movement (Matsuki et al. 2005). Thus, we investigated the relationship between the Hg lysis and Cl^- transport of rat parotid secretory granules. When the Cl^- ion in the medium was changed to gluconate, a substance impermeable to parotid secretory granule membranes, Hg lysis was completely blocked (black bar in gluconate; Fig. 2a). To elucidate the involvement of the channel in the chloride ion movement, we examined the effect of an anion channel blocker. AQP6 has been reported to be insensitive to known anion channel inhibitors such as DIDS, NPPB and DPC (Yasui et al. 1999a). When 1 mM DIDS was added to the secretory granule suspension, Hg lysis was partially blocked (Fig. 2b). We next evaluated the



In osmotic lysis of rat parotid granules	
Hg-sensitive component (a)	67.7 ± 3.7%
in Hg-sensitive component,	
-DIDS sensitive component (b)	31.7 ± 13.1%
-DIDS insensitive component (c)	36.1 ± 10.8%

Fig. 2 Hg^{2+}-induced granule lysis is coupled with chloride permeability. **a** In the absence of Cl^- ion by changing the suspension medium from KCl to K-gluconate, Hg^{2+}-induced granule lysis (*solid bars*) is completely blocked. **b** DIDS, an anion channel blocker (*black bar*), did not affect the control conditions (*open bar*). DIDS partially blocked the Hg^{2+}-induced granule lysis. The table on the right shows DIDS-sensitive (*b*) and -insensitive (*c*) components in Hg^{2+}-induced parotid secretory granule lysis. The 2 components were calculated as detailed in the Materials and Methods

contribution of AQP6 to Hg lysis. As shown in the table of Fig. 2b, the Hg^{2+}-sensitive component (67.7 %) was divided into the DIDS sensitive and insensitive components. These results suggest that the Hg lysis depends on DIDS sensitive (31.7 ± 13.1 %) and insensitive (36.1 ±10.8 %) Cl^- transporters.

Characteristics of Anion Transport in Rat Parotid Secretory Granule Membranes

To examine the involvement of AQP6 in parotid secretory granule lysis, the composition of the iso-osmotic solution was changed. AQP6 has been reported to be activated by low pH as well as by Hg^{2+} (Ikeda et al. 2002; Yasui et al. 1999a). At pH 4.0, the most AQP6-activating pH, Hg lysis could not be recorded because the secretory granule suspension turned cloudy (data not shown). To measure the acid sensitivity of AQP6, the solution pH was changed to basic (pH 8.0) or acidic (pH 6.0) and data were compared between the control and the Hg^{2+}-added group. In the Hg^{2+}-added group in pH 6.0, granule lysis was significantly facilitated compared with the control group (Fig. 3a). On the other hand, in the Hg^{2+}-added group in pH 8.0, granule lysis was slightly slower than control but not significant. We then changed the solution from Cl^- to NO_3^-, I^- or Br^-, which have been reported as permeable halide group anions in AQP6. Without Hg^{2+}, granule lysis was fastest in the I^- suspension, as reported previously (Gasser et al. 1990). When Hg^{2+} was added, granule lysis in the NO_3^- suspension was faster than in the Cl^-, I^- or Br^- suspensions (Fig. 3b). The order of Hg lysis rate was $NO_3^- > Br^- > I^- > Cl^-$. Taken together, anion movement in rat parotid secretory granules induced by Hg^{2+} is very similar to the AQP6 channel property.

Discussion

In this study, we used Hg^{2+}, a known activating drug for AQP6, to assess osmotic granule lysis and reveal the solute permeability of Hg lysis. Hg^{2+} clearly facilitated granule lysis at concentrations between 0.5 and 2 μM (Fig. 1a, b). Hg^{2+} is known to bind to cysteine residues of proteins (Raina et al. 1995). Furthermore, 2-ME, a protective reagent for sulfhydryl bonds in cysteine residues, is known to block the anion permeation induced by Hg^2 (Frigeri

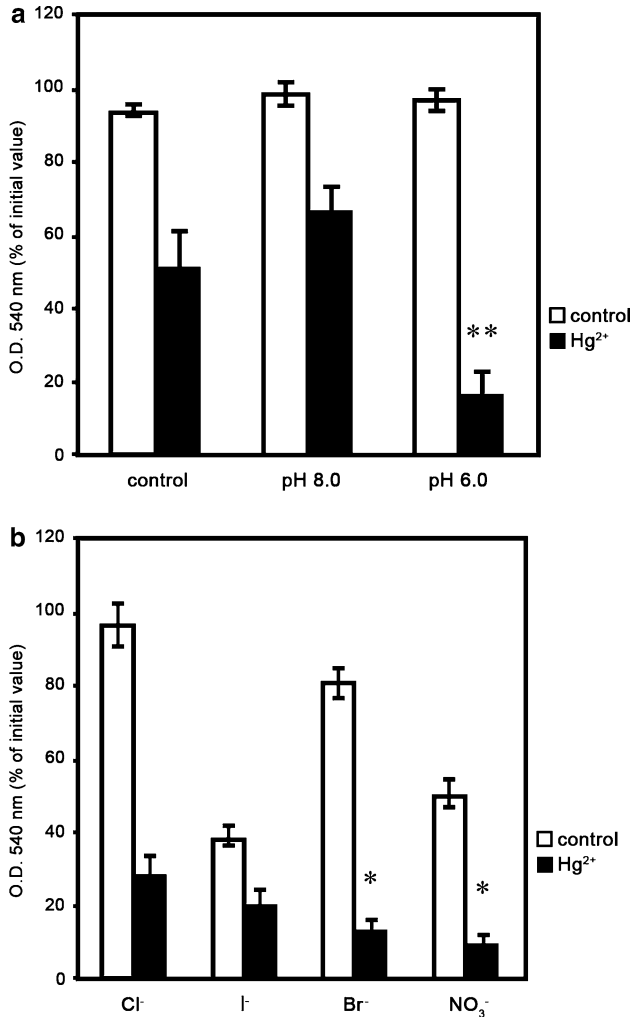


Fig. 3 Hg^{2+}-induced granule lysis is due to chloride transport via an acid activated, anion-permeable channel. Values are expressed as mean ± SEM ($n = 3$). Statistical significance was analyzed by repeated measures one-way ANOVA. Only significant data are indicated with an asterisk. **a** Hg^{2+}-induced granule lysis (*solid bars*) is facilitated by suspension at pH 6.0. **b** The sequence of halide group anion permeability in Hg^{2+}-induced granule lysis (*solid bars*) is $NO_3^- > Br^- > I^- > Cl^-$

et al. 1995). The inhibitory effect of 2-ME on Hg^{2+} was reproduced in Hg lysis (Fig. 1c). Therefore, these results suggest that Hg lysis is induced by the activation of proteins with cysteine residues in granule membrane proteins such as AQP6. Even without Hg^{2+}, by changing the composition of the medium from Cl^- to NO_3^-, secretory granule lysis was markedly facilitated (Fig. 3a). Moreover, Hg lysis was accelerated by acidic conditions (Fig. 3b). The sensitivity for acidic pH or NO_3^- is very similar to the AQP6 channel property that has been recorded by patch clamp techniques in AQP6-expressing oocytes or HEK293 cells (Hazama et al. 2002; Ikeda et al. 2002). Therefore, the Hg^{2+}-sensitive channel in the secretory granule membrane is anion permeable, and is likely AQP6.

The presence of anion conductance in rat parotid secretory granules has been previously demonstrated (Gasser et al. 1990; Gasser and Hopfer 1990; Gomes et al. 2009; Matsuki et al. 2005). When Cl^- was removed from the solution, Hg lysis was completely blocked (Fig. 2a). These data suggest that Hg lysis requires anion permeation. Furthermore, DIDS partially blocked Hg lysis (Fig. 2b). AQP6 was reported to be insensitive to known anion channel blockers, such as DIDS and DPC (Yasui et al. 1999a). Cystic fibrosis transmembrane regulator (CFTR) has been considered not to be localized in rat parotid secretory granules (Gasser and Hopfer 1990). DPC, a CFTR blocker, did not suppress the Hg lysis (data not shown). Therefore, it is likely that the DIDS-insensitive component of Hg lysis (indicated as component of (c) in Fig. 2b) is due to anion permeation through AQP6. These results suggest that DIDS divides Hg lysis into AQP6- (DIDS-insensitive) or other channel- (DIDS-sensitive) mediated solute permeation. Recently, a positive reaction was observed in the cytoplasm of parotid acinar cells of C57BL/6N mice using immunohistochemistry with an antibody against calcium-activated chloride channel 1/2 (mCLCA1/2), which is a DIDS-sensitive anion channel (Roussa et al. 2010). In Hg lysis, the contribution of mCLCA1/2 is negligible because the solution for Hg lysis contains EGTA (final concentration 1 mM) to chelate Ca^{2+}. Also, the solution for Hg lysis did not contain Ca^{2+}. Therefore, even if mCLCA1/2 is localized in parotid secretory granules, mCLCA1/2 does not participate in Hg lysis. Further studies are required to clarify the anion channel, which is sensitive to DIDS and Hg^{2+}, in granule osmoregulation.

Hg^{2+} is known as an activator of AQP6 (Hazama et al. 2002; Yasui et al. 1999a). Unfortunately, Hg^{2+} is not a specific inhibitor/activator of AQPs, and is also a nonspecific inhibitor of AQP5 (Lee et al. 1996; Raina et al. 1995). We have reported that AQP5 is involved in the osmoregulation of rat parotid secretory granule membranes (Matsuki et al. 2005). Therefore, it is necessary to consider whether the Hg^{2+}-induced granule lysis reflects an inhibitory effect of Hg^{2+} for AQP5 as well as an activation effect of Hg^{2+} for AQP6. However, the swelling of AQP5-expressing oocytes is inhibited by Hg^{2+} at a 100–300-fold higher concentration (300 μM–1 mM) (Lee et al. 1996; Raina et al. 1995) than the AQP6-activating concentration (1–10 μM). In our study, Hg lysis occurred at 1 μM Hg^{2+}, which is significantly lower than the concentration required to inhibit AQP5. Thus, it is likely that the Hg lysis is due primarily to the activation of AQP6. Further studies will be necessary to determine the contribution of AQP6 in the osmotic regulation of rat parotid secretory granules.

Because AQP6 was identified in acid secreting cells of the kidney, AQP6 has been considered to be involved in the

acid–base balance of the cytoplasm. Other renal AQPs, such as AQP2, localize in cytosolic small vesicles and translocate to apical membranes of principal cells in collecting ducts stimulated by vasopressin to permeate water. On the other hand, in response to an altered acid–base or water balance, AQP6 is not translocated to apical or basolateral membranes but the expression level of AQP6 mRNA and protein is increased (Promeneur et al. 2000). These results suggest that AQP6 not only maintains the acid–base balance but also the osmotic balance in the cytoplasm. Therefore, it is likely that AQP6 is involved in osmoregulation to maintain both the acid–base and osmotic balance of parotid secretory granules. Further experiments will be necessary to further clarify the role of AQP6 in parotid secretory granules.

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