High-resolution immunogold cytochemistry indicates that AQP4 is concentrated along the basal membrane of parietal cell in rat stomach

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Abstract Gastric parietal cells secrete hydrochloric acid in stomach. Because the secreted HCl solution is isotonic with the plasma fluid, it should accompany the water transport across the membranes of parietal cells. Aquaporins (AQPs) are water channel proteins that play the central role in the cellular handling of water in various mammalian tissues. Using immunocytochemistry, we found that AQP4 was expressed only in parietal cells of rat gastric mucosa. Immunogold electron microscopy study further demonstrated that AQP4 was mostly localized at the basal membrane of parietal cells. In the basal membrane, AQP4 was prominently enriched on the portion contacting with the basement membrane surrounding gastric glands. These results suggest that the contact between basement membrane and basal membrane may generate the signal involved in the targeting of AQP4 in gastric parietal cells.

Key words: Aquaporin; Gastric parietal cell; Gastric chief cell; Basal membrane; Immunohistochemistry; Immunogold electron microscopy

1. Introduction

Aquaporins (AQPs) are the membrane proteins that allow high permeability of water across the cell membrane and play the central role in the handling of water in various mammalian tissues, such as kidney, brain and lung [1]. So far, nine members of aquaporins (AQP1–9) have been identified [2–12]. Because expression pattern of each AQP in various tissues differs from one another, they may bear specific functional roles in each organ [1].

Gastric parietal cell secretes protons (H^+ ions) via H^+/K^+-ATPase which is localized on its apical and tubulovesicular membranes [13]. The extrusion of H^+ ions is associated with a concomitant excretion of Cl^- from the apical membrane, which results in secretion of the hydrochloric acid (HCl) from parietal cells. The concentration of HCl in the secreted solution was shown to be 0.17 N, which is isotonic with plasma fluid [14]. The mechanism whereby water is transported across the gastric epithelium in order to establish the isotonicity of the acid solution secreted by gastric parietal cells is not known. Recently, it was shown by using immunocytochemistry that AQP4 was expressed in parietal cells of rat stomach [15–17] and also in parietal and chief cells of human stomach [18], which suggests that AQP4 is involved in the water transport in parietal cells.

In this study we examined the subcellular localization of AQP4 in rat gastric mucosa by performing double staining of AQP4 with anti-H^+/K^+-ATPase antibody and also by using immunogold electron microscope technique. We found that AQP4 was localized mostly at the basal membrane in parietal cells. These results suggest that the roles of basal and lateral membranes of the parietal cells are different in the water transport.

2. Materials and methods

2.1. Antibody

The polyclonal antibody for AQP4 (LL182) was raised in rabbits against synthetic peptides corresponding to amino acids 280–296 and have been characterized previously [19,20].

2.2. Immunocytochemistry

Immunohistochemistry was performed as described previously [21]. Briefly, male Wistar rats weighing 200–250 g were deeply anesthetized with pentobarbital sodium (50 mg/kg i.p.), and stomachs were fixed by transcardiac perfusion with the following phosphate-buffered (1) or bicarbonate-buffered (2) fixatives: (1) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4; (2) 4% paraformaldehyde, pH 6.0, followed by 4% paraformaldehyde, pH 10.5 ('pH shift protocol'; 0.2% picric acid was added to both solutions).

Light microscopic immunocytochemistry (tissue fixed by fixative (1), cryoprotected in sucrose, and cut at 12 μm on a cryostat) was performed using a method of indirect fluorescence. Samples were washed three times with PBS for 10 min each, treated with pre-incubation solution (10% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100 in 0.01 M PBS, pH 7.4) at room temperature for 1 h, and then incubated with anti-AQP4 LL182 (1.0 μg/ml) and mouse monoclonal anti-H^+/K^+-ATPase (β-subunit) antibody (Affinity Bioreagents, Golden, CO, USA) in primary antibody solution (3% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide in 0.01 M PBS, pH 7.4) at room temperature overnight. The sections were washed three times with PBS at room temperature for 10 min each and visualized with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (EY Laboratories, San Mateo, CA, USA) and Texas Red-labeled anti-mouse IgG (Protos Immunoresearch, San Francisco, CA, USA) in secondary incubation solution (3% NGS, 1% BSA, 0.5% Triton X-100 in 0.01 M PBS, pH 7.4). The sections were examined with a confocal microscope (MRC-1024, Bio-Rad, Hertfordshire, UK).

For electron microscopic immunocytochemistry, after fixation perfused with the fixative (2), small blocks of the stomach were subjected to freeze substitution as described by Hjelle et al. [22]. In brief, the specimens were cryoprotected by immersion in graded concentrations of glycerol (10, 20, 30%) in phosphate buffer and plugged into liquid propane (−170°C) in a cryofixation unit (KF 80; Reichert, Wien, Austria). The samples were then immersed in 1.5% uranyl acetate dissolved in anhydrous methanol (−90°C) in a cryosubstitution unit

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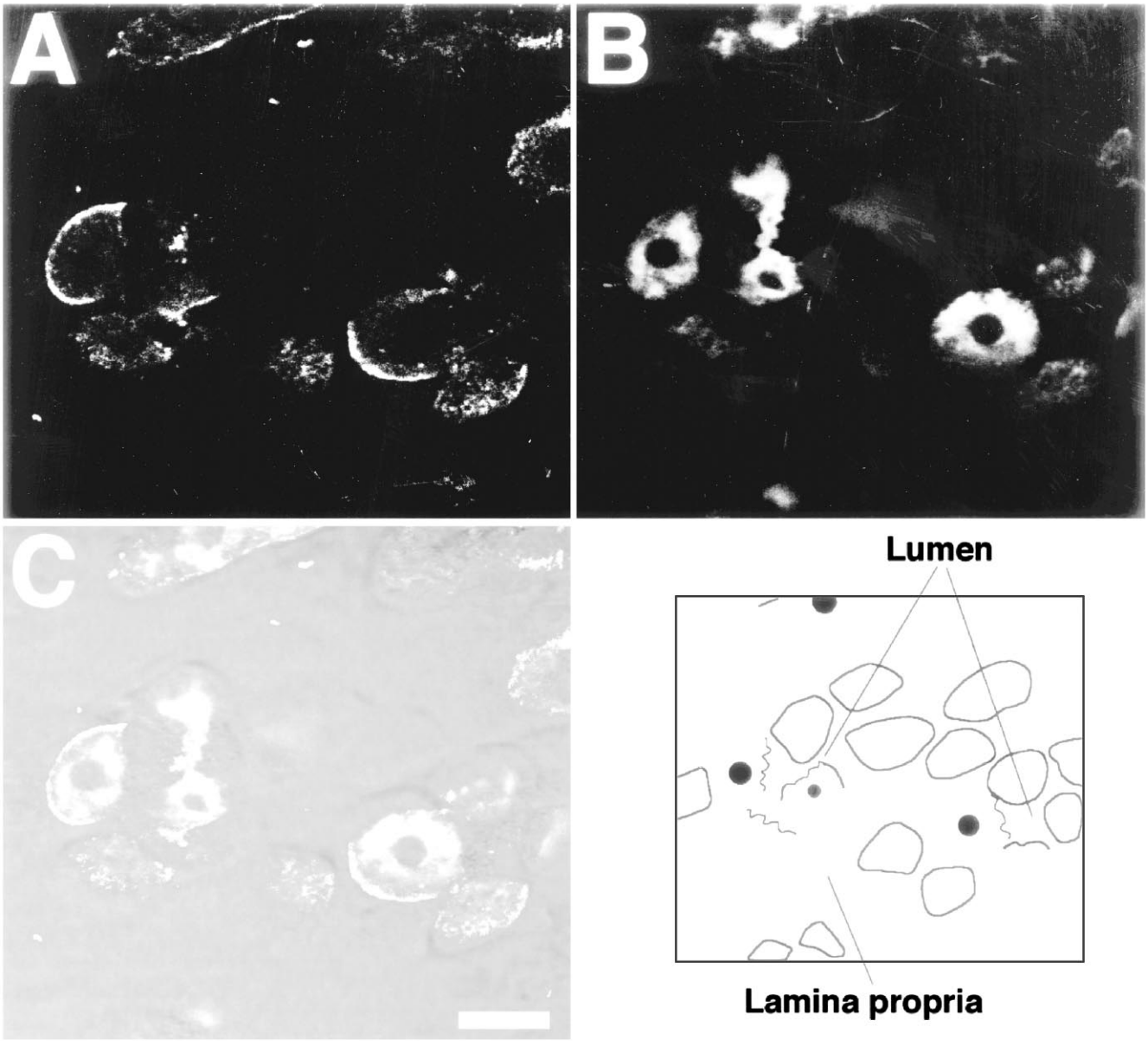

Fig. 1. Immunohistochemical analysis of AQP4 in the parietal cell. A sagittal section of rat stomach (12 μm) was double-stained with affinity-purified rabbit anti-rat AQP4 antibody, followed by FITC-conjugated anti-rabbit IgG (A and C, in green), and monoclonal anti-β-subunit of H^+/K^+-ATPase antibody, followed by Texas Red-labeled anti-mouse IgG (B and C, in red). C: Double exposures of both images. Scale bar (shown in C), 10 μm. The scheme of images is shown on the bottom right.

(AFS; Reichert). The temperature was raised in steps of 4°C/h to −45°C. Samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin to methanol. Polymerization was carried out with UV light (360 nm) for 48 h.

Ultrathin sections were cut with a Reichert ultramicrotome, mounted on nickel grids and processed for immunogold cytochemistry as described by Matsubara et al. [23]. Briefly, the sections were treated with a saturated solution of NaOH in absolute ethanol (2–3 s), rinsed in PB, and incubated sequentially in the following solutions (at room temperature): (1) 0.1% sodium borohydride and 50 mM glycine in Tris-buffer (5 mM) containing 0.1% Triton X-100 and 50 mM NaCl (TBST; 10 min); (2) 2% human serum albumin in TBST (10 min); (3) primary antibody (anti-AQP4, 2.0 μg/ml) diluted in the solution used in the preceding step (2 h); (4) same solution as in step (2) (10 min); (5) gold-conjugated Fab fragments (10 min; EM.GFAR10; BioCell Research Laboratories, Cardiff, UK), diluted 1:20 in TBST containing human serum albumin and polyethylene glycol (0.5 mg/ml, 2 h). Finally, the sections were counterstained and examined in HITACHI 7100-α electron microscope.

2.3. Quantification

We performed quantification and statistical analysis. Immersion-fixed material was used for quantitation and statistical analysis. The immuno-incubation was performed with anti-AQP4 (2.0 μg/ml) followed by conjugated Fab (10 nm).

Each picture represented a 4.1×2.7 μm rectangle at the level of the specimen. To avoid the effect of inadvertent differences in general labeling intensity, comparisons were made between membrane domains sampled from one representative section. All membrane segments were sampled at random. Membrane curve was calculated by hand.

The number of particles which were localized within 50 nm from their membrane were countered simply by hand and the number of particle densities per unit length of membrane was calculated.

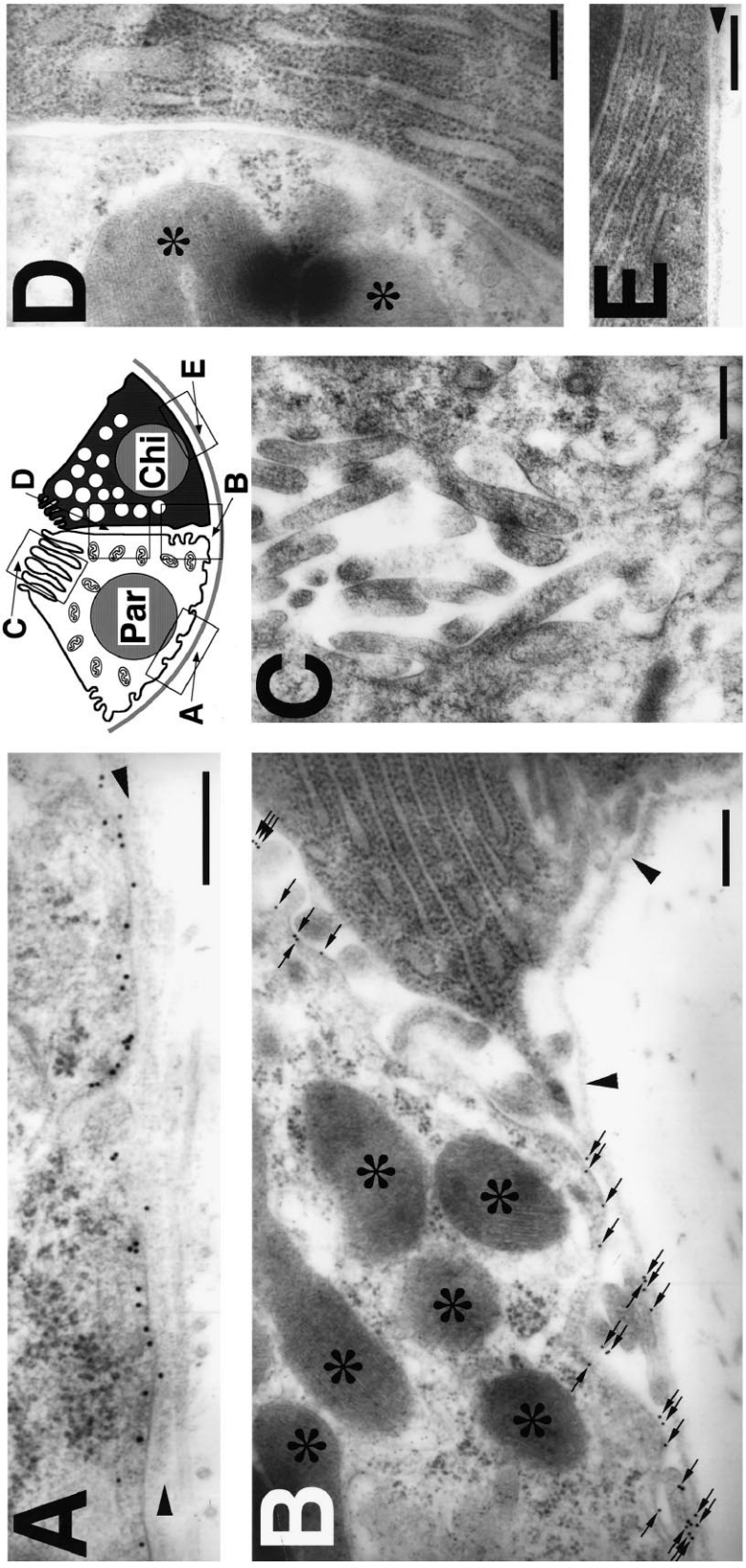

Fig. 2. Immunogold labeling of AQP4 in parietal and chief cells. Electron microscopic images of the portions as indicated in the bottom left scheme. The characters 'Par' and 'Chi' indicate parietal and chief cells, respectively. Immunogold labeling was detected basal (A, B) and the low part of lateral membranes (B) but not apical, tubulovesicular (C) and the higher part of lateral membranes (D) in parietal cell. No labeling was detected in any domain regions of chief cell (B, basal and lateral membranes; E, basal membrane). Small arrows indicate AQP4 labeling. Asterisks and arrowhead indicate mitochondria and basement membrane, respectively. Weak but apparent immunogold labeling was detected on the low part of lateral membrane (B). Scale bars, 0.5 μm.

3. Results

3.1. Double immunostaining of AQP4 and H^+/K^+-ATPase in rat gastric mucosa

A specific antibody to AQP4 has been characterized previously [19,20]. Fig. 1 shows immunohistochemical examination of a sliced section of rat stomach using the anti-AQP4 and anti-H^+/K^+-ATPase (β-subunit) antibodies. Fig. 1A and B are the immunostainings of anti-AQP4 and anti-H^+/K^+-ATPase (β-subunit) antibodies, respectively. Immunoreactivity of AQP4 (green) was detected on the basolateral membranes of the cells in glandular bodies of fundic glands (Fig. 1A), while that of H^+/K^+-ATPase (red) was in the cytosolic regions of probably the same cells. The double immunostaining shown in Fig. 1C clearly indicated that the immunoreactivities of AQP4 and H^+/K^+-ATPase (β-subunit) coexisted but were not overlapped in the same cells. These results suggest that AQP4 is localized on the basolateral membranes of parietal cell because H^+/K^+-ATPase has been shown to be localized on the apical and tubulovesicular membranes but not on the basolateral membrane [24]. Because the immunoreactivity of AQP4 was detected only in the cells which showed the reactivity to H^+/K^+-ATPase (β-subunit) (Fig. 1C), the chief cells may not express AQP4 in rat gastric mucosa [15–17].

3.2. Immunogold electron microscopic examination of AQP4 immunoreactivity

To clarify the subcellular localization of AQP4 in parietal cells, we performed immunogold labeling analysis with anti-AQP4 antibody. Gold particles of AQP4 immunoreactivities were concentrated along the basal membrane of parietal cell (Fig. 2A, B), but were not detected at the apical membrane and tubulovesicles (Fig. 2C). The gold particles were also not detected on the lateral membrane except its low part (Fig. 2B and D, see also the schemes in Figs. 2 and 3). The low part of the lateral membrane (LLM) possessed prominent infoldings as the basal membrane (BM), while most of the lateral membrane was free from the infoldings (Fig. 2A, B and D). Therefore, it seems likely that LLM can be recognized as the extension of the BM and has different properties from the main part of the lateral membrane (LM) (see the scheme in Fig. 3). The densities of gold particles on the various parts of cell membrane in parietal cells were examined as described in Section 2 (Fig. 3A). They were 8.3 ± 0.9 particles/μm on BM, 2.2 ± 0.4/μm on LLM, 0.3 ± 0.3/μm on LM, 0.2 ± 0.1/μm on AM. It is clear that gold particles are localized mostly on BM. They were also detected on LLM but the enrichment of gold particles on LLM was nevertheless much less pronounced than that on BM (Figs. 2B and 3A).

In double labeling experiments of AQP4 and H^+/K^+-ATPase (β-subunit) with gold particles of two different sizes, the different sized particles were not colocalized on any part of the membrane regions in parietal cells; labeling of H^+/K^+-ATPase (β-subunit) was detected only on apical and tubulovesicular membranes but not on basolateral membrane (data not shown). This agreed with the immunofluorescence result shown in Fig. 1C.

Also consistent with the results obtained with immunofluorescence (Fig. 1C), no immunogold labeling of AQP4 was detected on the basolateral (Fig. 2B and E) and apical membranes (not shown) of chief cells.

In BM, it was noticed that the gold particles were much more predominantly localized on the membrane portion facing to the basement membrane (BSM) than on the bottom of the infoldings which is distant from BSM (see Fig. 2A where arrow heads indicate BSM). To analyze this observation quantitatively, we further compared the density of gold particles on the membrane portion facing to BSM and that on the bottom of infoldings as shown in the scheme of Fig. 3C. It was 14.0 ± 0.9 particles/μm on the portion facing to BSM, and 2.5 ± 0.3/μm on the portion distant from BSM (Fig. 3C). In LLM where no BSM exists, the density of gold particles was 1.9 ± 0.9/μm on the outer half of the membrane and 2.5 ± 0.6/μm on the inner half. Therefore, it is suggested that the contact of BM with BSM may be somehow involved in the mechanism for the enrichment of AQP4 on BM.

4. Discussion

This study for the first time showed that the localization of

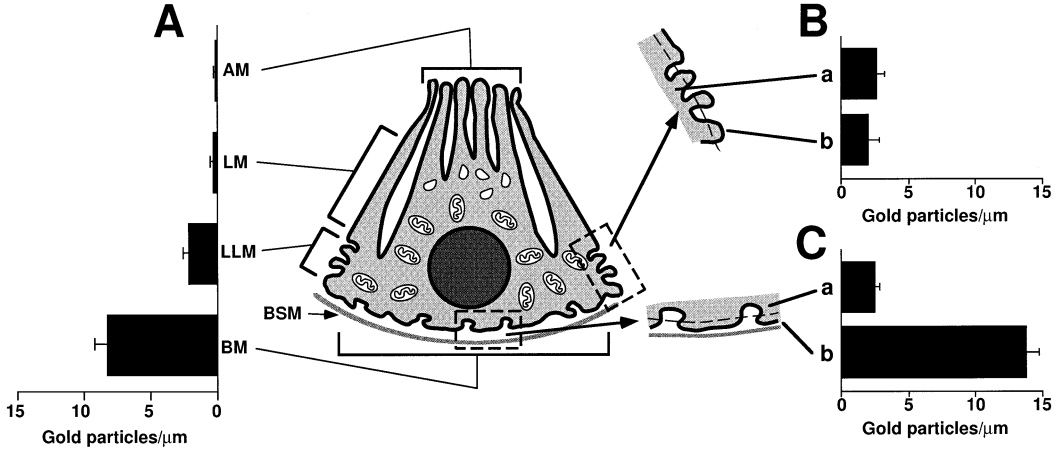

Fig. 3. Comparison of distribution of AQP4 immunoreactivity in the parietal cell membrane (A) and relationship between AQP4 labeling and contacting plasma membrane with the basement membrane (B, C). Values indicate number of gold particles per plasma membrane unit (1 μm). A: The following membrane domains were analyzed (n, number of observations): AM (apical membrane, $n = 15$), LM (lateral membrane, $n = 12$), LLM (low part of lateral membrane, $n = 9$) and BM (basal membrane, $n = 18$) of parietal cells. B: In LLM, comparison of the density of gold particles in the inner half of infolding (a) and in the outer half (b) ($n = 9$). C: In BM, comparison of the density of gold particles in the infoldings (a) and in the portion contacted with the basement membrane (b) ($n = 18$), as indicated in the scheme.

AQP4 was restricted essentially only to the basal membrane (Fig. 3). The lateral membrane was free from AQP4, except the low part (LLM) adjacent to the basal membrane (BM). Because the density of gold particles even at LLM was much lower than that of BM, it is suggested that some signal specific to BM is regulating the targeting of AQP4 to BM in parietal cells. The BM of parietal cells, which was enriched with gold particles, faced to the basement membrane (BSM, as indicated by arrow heads in Fig. 2), while the lateral and apical membranes did not. In BM, it was noticed that the gold particles were enriched much more prominently to the portion contacting with BSM than to that distant from BSM (Fig. 3C). Thus, the contact between the parietal BM and BSM might play an important role in enrichment of AQP4 at the basal membrane. Actually, in LLM where no BSM exists, the gold particles distributed diffusely along the infoldings (Fig. 3B). Actually, it was previously indicated that laminin in BSM may be involved in the control of specific clustered localization of AQP4 and Kir4.1 on retinal Müller cell membrane [25,26]. Therefore, similar mechanisms might underlie for the BSM-induced enrichment of AQP4 in the BM of parietal cells. Because AQP4 possesses the C-terminal tail sequence of LSSV, this suggests its interaction with PDZ domain-containing proteins, such as PSD-95/SAP90 and SAP97 [27]. The anchoring protein might be involved in the BSM control of subcellular localization of AQP4 in parietal cells. However, none of PSD-95, SAP97 or EBP50 could bind to AQP4 in vitro (data not shown). Further studies are needed to identify the anchoring protein for AQP4 and the signals of BSM controlling the targeting of the AQP4-anchoring protein complex to BM in parietal cells.

The gold particles for AQP4 were not detected at all on the apical or tubulovesicular membranes where we detected immunoreactivity of the H^+/K^+-ATPase. This anatomical evidence therefore suggests that AQP4 is not involved in the secretion of water from the parietal cell into the gastric lumen. However, the secretion of HCl solution should result in a loss of water from parietal cells and an increase of the osmolarity of the intracellular milieu of parietal cells. This, if uncorrected, would cause a decrease of cell volume. The presence of AQP4 proteins upon the basal membrane suggests that AQP4 may mediate water transport from the interstitial fluid to the intracellular milieu in order to maintain cellular osmolarity and volume during the secretion of HCl solution from the apical side of parietal cells. Although previous studies have indicated that the apical membrane of parietal cells possesses low permeability of water [28], the water transport across the apical membrane may be needed to secrete isotonic HCl solution from parietal cells. Because the expression of AQP3 and AQP8 mRNA were detected in stomach [8,11], these AQPs might explain the water permeability at the apical membrane of parietal cells. Further studies are needed to clarify the whole view of water transport system in parietal cells.

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