Expression of aquaporins and vasopressin type 2 receptor in the stria vascularis of the cochlea

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Recently, considerable evidence has been accumulated to support the novel view that water homeostasis in the inner ear is regulated via the vasopressin–aquaporin 2 (VP–AQP2) system in the same fashion as in the kidney. Indeed, multiple subtypes of AQPs including AQP-2 are reported to be expressed in the cochlea. However, the mechanism that underlies VP–AQP-2 mediated water homeostasis remains to be elucidated. In the present study, the localizations of AQP-1, -2, -3, -4, -5, -6, -7, -8, -9, and vasopressin type 2 receptor (V2-R) in the stria vascularis (SV) were molecular biologically and immunohistochemically examined to evaluate the role of the AQP water channel system in water homeostasis of the SV. A RT-PCR study revealed that AQPs and V2-R mRNA are expressed in the cochlea. As for their immunohistochemical localization, the AQP-2 protein is expressed on the basal side of the basal cells of the SV, and proteins of AQP-3 and V2-R are expressed on the apical side of the basal cells. AQP-7 and -9 proteins are expressed on the apical side of marginal cells. AQP-4, -5, and -8 protein expressions could not be detected in the lateral wall of the cochlea. From the present results, water flux in the SV is thought to be regulated at the level of the basal cells by vasopressin. Furthermore, such a distribution of AQP-2, -3, and V2-R suggests that VP–AQP-2 mediated water transport might work actively in the basal cells from perilymph towards endolymph containing AQP-1, -7 and -9.

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1. Introduction

The homeostasis of water in the inner ear is essential for maintaining the functions of hearing and equilibrium. Since the discovery of aquaporin (AQP) water channels (Agre et al., 1993), it has become clear that these channels play a crucial role in water homeostasis not only in the kidney but also in the inner ear. Indeed, expressions of proteins and/or mRNAs of AQPs in the inner ear have already been reported including negative reports (AQP-1: Stankovic et al., 1995; Sawada et al., 2003; AQP2: Kumagami et al., 1998; Beitz et al., 1999; Merves et al., 2000; Mhatre et al., 2002; Sawada et al., 2002; Fukushima et al., 2005; Zhong and Liu, 2003; AQP-3: Beitz et al., 1999; AQP-4: Takumi et al., 1998; Minami et al., 1998; AQP-5: Beitz et al., 1999; Mhatre et al., 1999; Löwenheim and Hirt, 2004; AQP-6: Fukushima et al., 2002; Lopez et al., 2007; Taguchi et al., 2008; AQP-7: Beitz et al., 1999; Huang et al., 2002; AQP-8: Beitz et al., 1999; Huang et al., 2002; AQP-9: Huang et al., 2002). However, previous reports have had many contradictions. Some data show that AQP-2 mRNA is present in rat cochlea (Mhatre et al., 2002; Sawada et al., 2002; Fukushima et al., 2004). In immunohistochemistry, Merves et al. (2000) showed AQP-2 protein in the inner ear during mouse development, and Mhatre et al. (2002) also showed not in the SV but in the structures bordering the cochlea of both rat and mouse including Reissner’s membrane, the organ of Corti, inner and outer sulcus cells and the spiral limbus. However, other studies found AQP-2 protein only in Reissner’s membrane (Zhong and Liu, 2003) or did not find it at all (Kumagami et al., 1998; Beitz et al., 1999). Focusing on the stria vascularis (SV), the expressions of AQP-1, -2, -3, -6, and -7 have been histochemically confirmed (Mhatre et al., 2002; Sawada et al., 2003; Fukushima et al., 2005; Zhong and Liu, 2003; Huang et al., 2002; Taguchi et al., 2008; Nishimura et al., 2002).
et al., 2008). It should be noted that the expression of AQP-2 mRNA is up-regulated by vasopressin and down-regulated by V2-antagonists (Sawada et al., 2002; Takeda et al., 2003). These results indicate that water homeostasis of the inner ear is regulated in part via the vasopressin–aquaporin2 (VP–AQP2) system in the same fashion as in the kidney. In the cochlea, the main site of VP–AQP2–mediated water homeostasis may be the SV. However, the detailed localization of AQPs and vasopressin type 2 receptor (V2-R) in the SV and the role of AQPs’ water channels in inner ear water homeostasis remain obscure. In this report, we examined the detailed localization of AQP-1, -2, -3, -4, -5, -7, -8, -9, and V2-R in the SV. First, the mRNA expressions of AQP1 and V2-R in the cochlea were checked with RT-PCR, and then their localization in the SV was observed using immunoelectron microscopy (immune EM) in addition to normal immunohistochemical observations. Then, their role in water homeostasis of the SV is discussed.

2. Materials and methods

2.1. Experimental animals

The animals used were young Wistar rats (200–280 g). Molecular, biological, and immunohistochemical studies were performed. The animals were deeply anesthetized with sodium pentobarbital (200 mg/kg, IP injection), perfused from the left ventricle with phosphate-buffered saline (PBS, 10 mM phosphate buffer salts, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and sacrificed.

The care and use of these animals was approved by the Kochi Medical School Animal Care and Use Committee, which conforms to The Animal Welfare Act and the guiding principles for animal care of the Ministry of Education, Culture, Sports, and Technology of Japan.

2.2. RT-PCR

The mRNA expressions of AQP-1, -2, -3, -4, -5, -7, -8, -9, and V2-R were investigated. After the rats had been sacrificed, the cochlea was extirpated, and the lateral wall of the cochlea was prepared under a stereomicroscope. The prepared tissue samples consisted of the spiral ligament, the SV, and the organ of Corti except the modiolus. Total RNA was extracted from two cochleae using the RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA using the SuperScript II kit (Invitrogen Corp., Carlsbad, CA, USA). A negative control was obtained by excluding reverse transcription. Primers for the detection of AQP and V2-R were designed on the basis of the published sequences from rats: AQP-1 (GenBank X67948), AQP-2 (GenBank D13906), AQP-3 (GenBank D17695), AQP-4 (GenBank U14007), AQP-5 (GenBank U16245), AQP-7 (GenBank AB000507), AQP-8 (GenBank AB005547), AQP-9 (GenBank AF016406), and V2-R (GenBank NM019136). The sequences of the primers and the expected band sizes are shown in Table 1. RT-PCR was performed using HotStar Taq (Qiagen, Hilden, Germany) and carried out using a thermal Cycler (TP9000, Takara, Ohtsu, Shiga, Japan). The procedure was performed as follows: an initial denaturing period of 15 min at 95 °C, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension period of 10 min at 72 °C. PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide, before being directly sequenced using ABI PRISM 310 (PE Applied Biosystems, Foster City, CA, USA) with the Big Dye® terminator v3.1 sequencing kit (PE Applied Biosystems).

2.3. Immunohistochemistry

We used two methods for the tissue preparation. As the first method, after PBS perfusion, 4% paraformaldehyde (PFA, pH 7.4) in PBS was intravenously perfused as well. The temporal bones were dissected and immersed in the fixative overnight and then decalcified in 0.12 M ethylenediaminetetraacetic acid (EDTA) in PBS (pH 7.4) for 7 days. The temporal bones were then finally soaked in sucrose in PBS (10% 4 h, 15% 4 h, then 20% overnight) and were then embedded in Tissue Tek OCT Compound (Sakura Finetechinal Co. Ltd., Tokyo, Japan). As the second method, the perilymphatic space was immediately perfused with the fixative containing 4% PFA and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The cochlea was removed and immersed in the same fixative for 1 h at 4 °C. Then, the cochlear duct lateral wall of the basal and second turn was dissected in the fixative, and tissue strips were embedded in Tissue Tek OCT Compound.

These tissues obtained as above were cut with a cryostat into slices (HM 505E, MICROM) of 10 μm thickness and collected on slides. The sections were pre-incubated for 1 h in a solution containing 10% normal goat serum to block nonspecific binding sites. The specimens were then exposed overnight at 4 °C to rabbit antibodies specific for AQP-1 (diluted 1:100, # AB3065, Chemicon, Temecula, CA, USA), -2 (diluted 1:100, # AB3274, Chemicon, Temecula, CA, USA), -3 (diluted 1:80, # AB3276, Chemicon, Temecula, CA, USA), -4 (diluted 1:200, # AB3594, Chemicon, Temecula, CA, USA), -5 (diluted 1:100, # AB3069, Chemicon, Temecula, CA, USA), -7 (diluted 1:50, # AB3075, Chemicon, Temecula, CA, USA), -8 (diluted 1:100, # AB3077, Chemicon, Temecula, CA, USA), -9 (diluted 1:80, # AB3079, Chemicon, Temecula, CA, USA), and V2-R (diluted 1:100, # AB1797, Chemicon, Temecula, CA, USA). The sections were then incubated for 6 h at room temperature in Alexa 546 goat anti-rabbit IgG (diluted 1:100, # A-11035, Molecular Probes Inc., Oregon, USA). The sections were washed extensively with PBS, before being examined using an Axiovert 200M controlled by Axiovision LE software.

2.4. Immunoelectron microscopy

The tissue obtained using the second immunohistochemical method was embedded in LR white resin (London Resin Company Ltd., London, England). Thin sections were cut perpendicular to the apical surface of the marginal cells (MC) on an Ultracut E ultramicrotome (C. Reichert AG, Vienna, Austria) and used for immuno-

Table 1

<table>
<thead>
<tr>
<th>Band size</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tbody>
<tr>
<td>AQP-1 534 bp</td>
<td>TCACATCACTGCGTAGTCACTACCA</td>
<td>GTCACACCTTCATCGCGCTCTC</td>
</tr>
<tr>
<td>AQP-2 447 bp</td>
<td>TGGTCTTTGGGCGACGCTTTT</td>
<td>ATGCACGGACAGGGAGTAC</td>
</tr>
<tr>
<td>AQP-3 495 bp</td>
<td>GCCGCCCTTGGGGCCATCGCTT</td>
<td>ACCACGCTTCTGCAAGCTT</td>
</tr>
<tr>
<td>AQP-4 459 bp</td>
<td>GCGGCGGCTGGGTTAGTAGTGC</td>
<td>CCTAGGAGGGAGTAGAGTT</td>
</tr>
<tr>
<td>AQP-5 360 bp</td>
<td>GCCGCCACATCAAGCAGCATC</td>
<td>GTGTCGAGAGGAGTAAGTAG</td>
</tr>
<tr>
<td>AQP-7 338 bp</td>
<td>AGTCTCCTCCGCGAGGTCGATAC</td>
<td>CACACACAGTCTCCACTGAGT</td>
</tr>
<tr>
<td>AQP-8 543 bp</td>
<td>TGGTGAAGGGAGACACTCGCAG</td>
<td>CACCAAGAGAGGAGTACAG</td>
</tr>
<tr>
<td>V2-R 684 bp</td>
<td>ATGGGCTGGGCAGTATGCGCTC</td>
<td>CACACACAGTCCACGAGC</td>
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The initial denaturing period of 15 min at 95 °C, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension period of 10 min at 72 °C. PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide, before being directly sequenced using ABI PRISM 310 (PE Applied Biosystems, Foster City, CA, USA) with the Big Dye® terminator v3.1 sequencing kit (PE Applied Biosystems).
3.1. RT-PCR

Fig. 1 shows the RT-PCR products after 35 cycles. AQP-1, -2, -3, -4, -5, -7, -8, -9, and V2-R mRNAs were expressed in the rat cochlea. The PCR products were of the expected size as shown in Table 1. The nucleotide sequences of the bands agreed completely with the known sequences of rat AQPs and V2-R. No PCR product was obtained when RT was omitted (negative controls).

3.2. Immunohistochemistry

V2-R was expressed in the basal cells (BC) of the SV (Fig. 2c and d). AQP-1 was clearly expressed in type 3 fibrocytes and diffusely expressed in the SV (Fig. 2b). AQP-4, -5, and -8 proteins were not expressed in the SV (Fig. 2e and f). Concerning AQP-4, -5, and -8, the results of RT-PCR and immunohistochemistry were incompatible. AQP-4 protein was expressed in Hensen’s cells, Claudius cells, and the inner sulcus cells (Data not shown). AQP-5 was observed in the external sulcus (ES) cells and cells of the spiral prominence (Data not shown). Therefore, PCR products of AQP-4 and -5 seem to reflect the expressions of these proteins in cochlear tissues other than the stria vascularis. AQP-8, however, was not detected anywhere. AQP-7 protein expression was observed on the apical side of the MC and BC of the SV (Fig. 2g). AQP-9 protein expression was observed on the apical side of the MC of the SV (Fig. 2i). Moreover, we performed the pre-absorption test on AQP2. There was no expression on the basal cell of the SV (Data not shown).

3.3. Immunoelectron microscopy

SV contains three cell types, MC, intermediate cells (IC) and BC. MC is constructed from one superficial layer and are closely combined by tight junctions. BC consist of a few layers, are combined closely by tight and gap junctions, and have radiating processes that interdigitate with the processes of IC and MC. The BC also have ascending processes that surround and partially isolate the basal processes of the MC.

The plasma membrane of BC in the SV was labeled with immunogold against AQP-2, -3, and V2-R antibodies. AQP-2 was detected on the basal membrane of the BC and the vesicles in the BC, but not on the apical membrane (Fig. 3a–c). AQP-3 was detected on the apical side of the radiating processes of the BC (Fig. 4a–c), but not on the basal membrane. V2-R was observed on the apical side of the radiating processes, but not on the basal side of the BC (Fig. 5a–c).

The plasma membrane of the IC was labeled with immunogold against AQP-1 (Fig. 6a–c), as shown in our previous report (Sawada et al., 2003).

Both AQP-7 and AQP-9 were labeled in the apical region of the MC (Figs. 7a, b and 8a, b). AQP-7 labeling was frequently observed in the vesicles immediately beneath the apical membrane, not on the membrane. AQP-7 was also diffusely detected in the BC, not on the membrane (Fig. 7c).

Meanwhile, immunogold AQP-9 labeling was observed on the plasma membrane of the apical side or on the vesicles beneath the plasma membrane.
4. Discussion

The ultrastructural localizations of AQP5s in the inner ear have been already reported for AQP-1 and -6. Takumi showed by immunocytochemical studies including immunogold analysis that AQP-1 is expressed along the plasma membranes of type III fibrocytes in the spiral ligament and of sub-basilar tympanic cells. However, their light microscopic immunocytochemistry detected no cells that were labeled for AQP-1 in the SV. On the other hand, our previous study reported that AQP-1 protein is expressed not only in the spiral ligament but also in the SV (Sawada et al., 2003). Our previous immunoelectron microscopic study revealed that immunogold particles were found along the plasma membranes of the IC. In the present study, these results were reconfirmed. As for the expression of AQP-6 in the SV, it was located in the membrane of the intracellular vesicles of the SV. From the lack of AQP-6 expression on the plasma membrane, AQP-6 was thought not to have a direct role in water flux via the plasma membrane (Taguchi et al., 2008). In addition to the expression of AQP-1 in SV, the present light microscopic immunofluorescence studies revealed that AQP-2, -3, -7, -9, and V2-R are clearly expressed in the SV. Since SV is thought to be the main site of active ion and water transport in the endolymph (Sterkers et al., 1988), it seems to be reasonable that multiple subtypes of aquaporins are expressed there. Specifically, it has been noted that the expression of AQP-2, -3, and V2-R was localized in the BC, at the boundary between the perilymphatic system and the endolymphatic system. Such a distribution of AQP-2, -3, and V2-R suggests that VP–AQP-2 mediated water transport might work actively in the BC.

The present study is the first report to define the ultrastructural localization of AQP-2, -3, -7, -9, and V2-R in the SV. Their distribution on or near the plasma membrane is shown in Fig. 9. AQP-3 and V2-R were localized at the apical side of the BC, while AQP-2 was located along the basal membrane of the BC. In renal collecting duct principal cells, AQP-2 is known to be located on the opposite side to V2-R, and AQP-3 on the same side as V2-R (Nielsen et al., 2002; Robben et al., 2004). Although this distribution pattern is not always confined to the SV, the present study supports that AQP-2, -3, and V2-R are distributed in the same manner as in the kidney. According to Sziklai et al. (1987), VP may exert its effects via the perilymphatic side of the SV. If so, V2-R might be localized at the lateral side of the BC. However, V2-R is located at the apical side. Therefore, VP is thought to act on the BCs via the intrastrial artery.

It is known that VP exerts its effects on the target organ by binding to its receptor, V2-R. The present results suggest that the BC is the target organ of VP and that VP–AQP-2 mediated water transport occurs actively in the BC toward the endolymphatic system. Since the driving force of AQP water channels is the osmotic gradient and the osmolarity of the endolymph is significantly higher than that of the perilymph (Konishi et al., 1984; Sterkers et al., 1984), the water in the perilymphatic compartment might enter into the intracellular space of the BC via AQP-2 water channels, and then the water in the intracellular space of the BC might exit into the extracellular space of the SV (intrastrial space) via AQP-3.

The other possible water pathway is water flow via gap junctions. Orce et al. reported that a gap junction inhibitor significantly reduced osmotic water flow (Orce et al., 2004). In the SV, the BC
Fig. 3. AQP-2 labeled with immunogold in the SV. AQP-2 labeled with immunogold was observed in the BC, as seen in the higher magnification views (b and c) of the areas enclosed by rectangles in the lower magnification view (a). AQP-2 is positive within the cytoplasm of the BC (b, arrows) and at the basement membrane (c, arrowheads), but not on the apical (intrastral) side of the plasma membrane. Scale bar: 0.5 μm in (a), 0.1 μm in (b) and (c) (MC, marginal cell; BC, basal cell; Cap., capillary; V, vesicle).

Fig. 4. AQP-3 labeled with immunogold in the SV. AQP-3 labeled with immunogold was observed in the BCs, as seen in (b) and (c) (higher magnification views of the areas enclosed by rectangles in the low magnification view of SV (a)). AQP-3 is positive at the apical membrane in the radiating processes (b, arrows), but not on the basement membrane (c). Scale bar: 0.5 μm in (a), 0.1 μm in (b) and (c).
and IC are coupled by the gap junction system. Gap junction channels have a pore with a diameter of 1.0–1.5 nm, permitting intercellular passage of particles with a molecular weight of less than 1000, for example, ions, nucleotides, siRNAs, dyes, and inositol phosphates (Zhao et al., 2006). If gap junction channels permit water passage (Verselis and Brink, 1986), water may flow into the intrastrial spaces from the IC via AQP-1 since AQP-1 is expressed in the IC (Sawada et al., 2003, present study). Furthermore, the BC connect the fibrocytes of the spiral ligament via the gap junction system. This connection permits water influx into the BC via AQP-1 water channels on type III fibrocytes of the spiral ligament. Therefore, the syncytium, coupled with fibrocytes, BCs, and ICs via the gap junction system, is thought to function as the first barrier to water flow from the perilymphatic to endolymphatic compartments. More studies are required to elucidate these mechanisms.

Here, one question arises: via what pathway does water flow out from the extracellular space and into the scala media? The most probable candidate is a route through the MC. Actually, two subtypes of water channels, AQP-7 and AQP-9 are expressed in the apical region of the MC, as shown in Figs. 7b and 8b. The localization of AQP-7 is compatible with Huang’s results (Huang et al., 2002). However, AQP-7 labeling was detected on the vesicles immediately beneath the apical membrane in the MC, but not on the membrane in the immune EM. Recent reports have shown that AQP-s are present in cytoplasmic vesicles in human parotid aciner cells (Smith et al., 1999) and the mouse liver (Ferri et al., 2003), and intracellular vesicles in the rat kidney (Yasui et al., 1999).
Transport vesicles are in part involved in endocytosis and exocytosis (Burgoyne and Morgan, 2003). Indeed, an application of VP produces an increase in vesicle formation in MC (Nishimura et al., 2008). These results indicate that the AQP-7 observed on the vesicle immediately beneath the apical membrane is related to exocytosis as a water transport route under VP regulation. Recently, AQP-7 has been reported to be permeable not only to water but also to glycerol and so-called aquaglyceroporins (AQP-3, -7 and

**Fig. 7.** AQP-7 labeled with immunogold in the SV. Immunogold AQP-7 labelings were observed in the MC and BC, as seen in the higher magnification views (b and c) of the areas enclosed by rectangles in the lower magnification view (a). In the MC, AQP-7 was positive on the vesicles immediately beneath the plasma membrane of the apical membrane (b, arrowheads), not on the membrane. In the BC, AQP-7 was positive in the cytoplasm (c, arrows). Scale bar: 0.5 μm in (a), 0.1 μm in (b) and (c).

**Fig. 8.** AQP-9 labeled with immunogold in the SV. Immunogold AQP-9 labelings were observed in the MC, as seen in the higher magnification views (b) of the area enclosed by a rectangle in the lower magnification view (a). AQP-9 was positive in the apical region of the MCs (b and c, arrows). (c) Another place of the MC. Scale bar: 0.5 μm in (a), 0.1 μm in (b) and (c).
In view of the inference described above, the following routes of water flux with two barriers can be proposed on the basis of the assumption that osmolarity increases gradually in the direction from the perilymphatic compartment to the endolymphatic compartment, although the osmotic gradient within the SV remains to be elucidated. The first barrier between the perilymphatic and endolymphatic compartments is composed of the syncytium of the fibrocytes, BC, and IC, coupled with the gap junction system. According to the osmotic gradient, water flows into the syncytium through the AQP-1 channels on the type III fibrocytes (Fig. 9, line 1) or AQP-2 water channels on the BC (Fig. 9, line 2), and flows out into the intrastrial space through AQP-3 water channels on the BC (Fig. 9, line 3) or AQP-1 on the IC (Fig. 9, line 4). Generally, the driving force of AQP water channels is generated by the combination of trans-epithelial hydrostatic and osmotic pressure. Therefore, the amount of water moving per unit of time and surface is dependent on the osmotic or hydrostatic gradient and the AQP concentration on the epithelium (Parisi et al., 1997). AQP-1, -2, and -3 expression in the kidney is known to be directly and secondarily mediated by vasopressin and corticosteroids (Nielsen et al., 1999, 2002). In the inner ear, AQP-1 and -2 expression is mediated by corticosteroid and VP, respectively (Fukushima et al., 2002; Sawada et al., 2002). The water inlets and outlets of the syncytium are likely to be under hormonal regulation.

The second barrier is the MC. Water, which flows into the intrastrial space from the syncytium, is pumped up into the MC coupled with K⁺ ions through the Na⁺–K⁺–Cl⁻ cotransporter, which is abundantly expressed on the basolateral side of the MC (Fig. 9, line 5), and then, the water in the MC flows out into the endolymphatic space through AQP-9 water channels (Fig. 9, line 6) and/or AQP-7 water channels (Fig. 9, line 7).

The above-proposed water flux mechanism is based on two hypotheses (1) the osmolarity within the SV increases gradually in the direction from the perilymphatic compartment to the endolymphatic compartment and (2) the cotransporter works as a water pump (Loo et al., 1996; Zeuthen and MacAulay, 2002; MacAulay et al., 2004). Furthermore, expression of AQP-10, -11, and -12 in the SV was not examined in the present study because of the lack of the commercially available antibodies with adequate sensitivity. We cannot categorically deny that these AQPs are expressed in the SV, especially, in the basolateral (intrastrial) side of the MC, or that they play a role in water flux in the SV; however, the experimental findings that application of VP or loop diuretic induces enlargement of the intrastrial space (Nishimura et al., 2008; Higashiyama et al., 2003) seems to support the present proposed water flux mechanism. Further study is required to establish the water flux mechanism of the lateral wall of the cochlea.

Acknowledgments

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