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Identification of Aquaporin 5 (AQP5) within the Cochlea: cDNA Cloning and *in Situ* Localization

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Dysfunction of fluid and electrolyte homeostasis is considered to cause variety of inner ear disorders. One group of candidate proteins that may play a critical role in the inner ear fluid homeostasis is the aquaporins, a family of proteins whose members have well defined roles in fluid transport in variety of organs. This study reports the identification of AQP5, a member of the aquaporin family, within the rat inner ear and its *in situ* localization. AQP5 was initially identified within rat cochlear RNA via RT-PCR and sequence analysis of the amplified fragments. Immunoblot of cochlear homogenate yielded a predominant AQP5-immunoreactive band of M_r 35 kDa. The anti-AQP5 immunoreactivity, indicating expression of the AQP5 polypeptide, was localized within the cochlea *in situ* to the cell types that form the lateral wall of the cochlear duct—the external sulcus (ES) cells and the cells of the spiral prominence. Expression of AQP5 was observed in the apical turn but not the basal turn of the cochlea; nor was it observed in the vestibular neuroepithelia or its supporting cells. The restricted expression of AQP5 to the apical turns of the cochlea suggests its potential role in low frequency hearing.

Homeostasis of inner ear fluids and electrolytes is essential for normal function of the auditory and vestibular end organs. Collectively, these fluids transmit the mechanical stimulus of sound waves or motion to the sensory cells of the auditory and the vestibular organs and their physiochemical properties permit the sensory cells to transduce the incoming mechanical signal into electrical impulses. The inner ear architecture serves to compartmentalize two distinct types of circulating fluids designated as endolymph and perilymph (1). The endolymph, sequestered within a closed membranous chamber, the scala media, has high and low concentrations of K^+ and Na^+, respectively. The perilymph, sequestered within membranous chambers scala tympani and scala vestibuli joined at the apex of the cochlea, has high and low concentrations of Na^+ and low K^+, respectively, composition that is similar to the plasma. The distinct composition of these two fluids translates into a positive potential of 80 mV between the endolymph and perilymph. Dysfunction in the homeostasis of solute concentrations, osmotic/electrical gradients between the fluid filled scalae and total volume of the inner fluids is considered to be the cause of variety of inner ear disorders and concomitant hearing loss.

A critical homeostatic component of the inner ear fluids is considered to be active transport of water via aquaporins (AQP) or water transport proteins. Several members of the AQP gene family, designated AQP0 to AQP9, have been identified and their expression observed in almost every mammalian tissue (2, 3). Dysfunction of these proteins may potentially cause fluid and electrolyte disturbance in the body resulting in tissue/organ disorder (4, 5). As of now, mutations in three members of the AQP gene family, AQP0, 1 and 2, have been identified and characterized. Mutations of AQP2, initially documented to be expressed exclusively within the kidneys, have been described as the pathogenic cause of congenital nephrogenic diabetes insipidus (6, 7). AQP0 constitutes 60% of the total membrane protein in the lens fiber cells. Its deficiency and dysfunction as a consequence of mutations in the AQP0 gene has been associated with cataracts and presbyopia in mice (8). Mutations of AQP1 have also been reported; however, their effect is considered to be benign (9). However, with a possible singular exception (10) none of the mutations in AQP0, 1 and 2 has been

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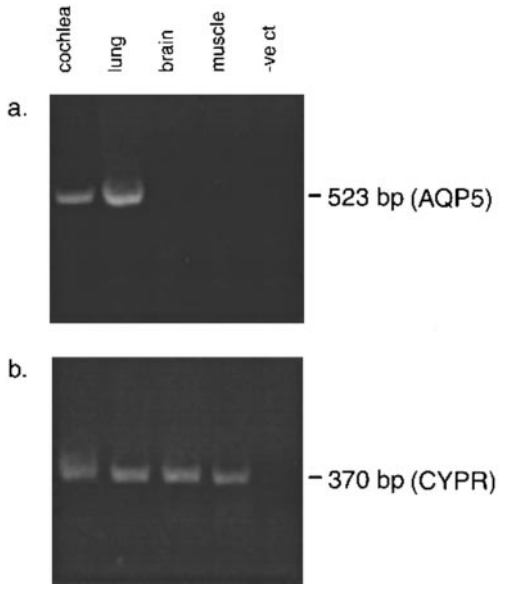

FIG. 1. RT-PCR analysis of AE2 expression in rat cochlear cDNA. cDNA from several tissues including the cochlea was assayed for AQP5 expression via PCR using AQP5-specific primers. (a) Both lung and cochlear cDNA yield the expected 523 bp fragment amplified using AQP5-specific primers. Brain and muscle, known not to express AQP5, are negative. (b) cDNA from all tissues tested for AQP5 expression are positive for expression of cytochrome P450 reductase (CYPR), a housekeeping gene using CYPR specific primers that yield the expected 370 bp fragment.

reported to affect the auditory function in the affected individuals.

The importance of fluid regulation to the inner ear function and the well documented role of the aquaporins in active water transport, has resulted in publication of several reports documenting expression of specific AQPs in the inner ear. Thus far, several members of the AQP gene family, including AQP1, 2, and 4, have been identified within the inner ear and their expression established in specific cochlear tissue types. AQP1 is the most ubiquitously expressed of all AQPs and the first member of the family to be characterized for its water transport function. Within the cochlea, AQP1 has been identified largely within type III fibrocytes in the spiral ligament (11). AQP2, the vasopressin regulated water channel is expressed predominantly in the kidneys, within the renal collecting ducts. Within the inner ear, AQP2 was found to be expressed in the epithelium of the endolymphatic sac, considered to be important in regulating volume of the endolymph (12). AQP4 is abundantly expressed in the brain, largely within the glial cells; it is also expressed in the kidney and the lung. Within the cochlea, expression of AQP4 has been localized in supporting epithelial cells, including Hensen's and the inner sulcus cells but not within the sensory cells. AQP4 and AQP4-mRNA was also detected in the central part of cochlear and vestibular nerves (13). The presence of these AQPs and their distribution pattern within the cochlea has led to speculation that they have a critical role in regulation of the inner ear fluids and hence the auditory function. However, the physiological role of AQP1, 2, and 4 to our hearing remains yet to be determined.

This study reports the identification and *in situ* localization of aquaporin 5 (AQP5), a member of the AQP family. AQP5 was initially cloned from the rat submandibular gland (14). Subsequently, AQP5 expression has been localized in lacrimal glands, cornea, trachea, lung and other salivary glands including the parotid and sublingual glands (14; Dibas *et al.*, 1998). This tissue distribution pattern suggests an important role of AQP5 in secretion of saliva, tears and pulmonary fluids. The human *AQP5* has been localized as a single copy gene at chromosome locus 12q13 and is in closely spaced tandem arrangement between AQP2 and AQP6 genes in an aquaporin gene cluster that also contains *AQP0* (15). AQP5 shares significant amino acid homology to AQP2, and its cytoplasmic loop D contains a protein kinase A consensus site also similar to AQP2 (15). The rat AQP5 gene is 91% identical to its human orthologue and has a mercurial osmotic sensitivity similar to other aquaporins (Delporte *et al.*, 1996).

AQP5 expression and localization within the rat inner ear, as established in this study, raises questions concerning its potential role in cochlear function and dysfunction.

MATERIALS AND METHODS

RNA isolation. Total RNA was isolated from cochleae and multiple other organs, all harvested from adult male Sprague Dawley rats. To obtain cochlear RNA, rats were sacrificed by overdose of sodium pentobarbital (250 mg/kg IP), temporal bones from both sides were removed, and membranous cochleae (2–4 mg each) were care-

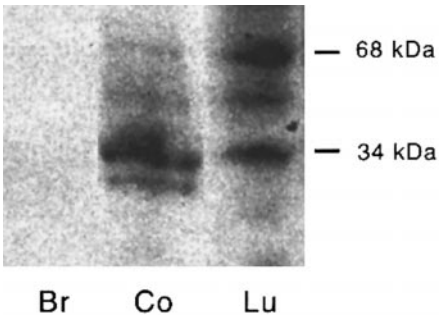

FIG. 2. Western blot analysis of AQP5 expression in the rat cochlea. Homogenate from several rat tissues including the cochleae were resolved on a 8% SDS-PAGE and then transferred to nitrocellulose membrane. The Western blot was probed with anti-AQP5 Abs and then developed using the ECL kit from Amersham. A predominant immunoreactive band of 34 kDa accompanied by a minor band of 28 kDa is observed in cochlear homogenate while two codominant bands, 34 and 68 kDa, are seen in the homogenate harvested from the lung (Lu). Brain (Br) homogenate is negative for anti-AQP5 immunoreactivity.

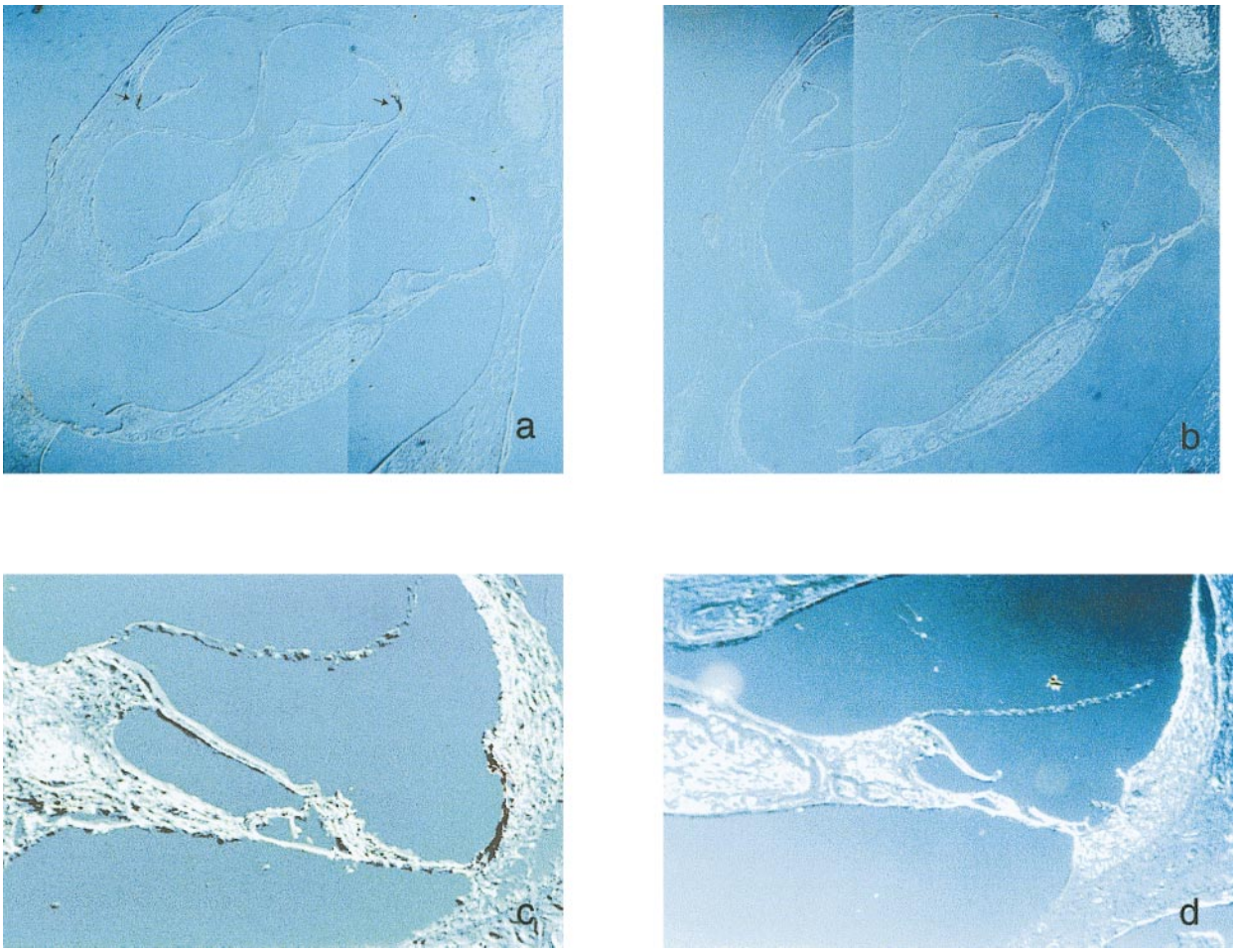

FIG. 3. Immunolocalization of AQP5 in the rat cochlea. Radial sections of the rat cochlea were hybridized with anti-AQP5 antibody and then developed with biotinylated, Alkaline phosphatse linked amplification system and stained with NBT/BCIP. Cell types, including the external sulcus cells and the spiral prominence, within the lateral wall of the cochlear duct in the upper turns of the cochlea are immunoreactive towards the AQP5 Ab, indicated by arrows (a). Cochlear sections are unstained when coincubated with the antibody in presence of competitive peptide (b). A higher power view of the cochlear ducts from the apical turn (c) and from the lower turn (d) seen in (a) illustrates the restricted expression of AQP5.

fully microdissected from the bony shell. The small cochlear size (2–4 mg) required pooling of 16 cochleae. Other organs, including brain, lung, and muscle were also collected. Total RNA was isolated from the harvested organs by the method of Chomczynski and Sacchi (2). Tissues were homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, extracted with phenol:chloroform:0.2 M sodium acetate (1:0.2:0.1), precipitated with isopropanol, washed, vacuum-dried and stored at −70°C in ethanol.

RT-PCR assay of AQP5 expression. One μg of total RNA harvested from individual tissues was reverse transcribed into cDNA using MMLV reverse transcriptase (2.5 units) (Gibco-BRL) and random hexamers (50 ng) (Gibco-BRL). in a total volume of 50 μl. Two μl of the RT product was then assayed for presence of AQP5 cDNA via PCR using rat AQP5-specific primers pairs A1B1 and A2B2, spanning 550 and 625 bp of AQP5 sequence (A1, GCTCCTTTTTGTCTTCTTTG; B1, TTCCTCCCAGTCGGTGTCG; A2, CAATGCGCTGAACAACAACACA; and B2, TGGGGAGGGGTGCTTCAAACT). PCR was carried out in PTC 200 (MJ Research) in a total volume of 20 μL with 1.25 mM $MgCl_2$, 200 μM dNTP, 5 picomol of forward and reverse primers and a unit of *Taq* DNA polymerase (BRL). The temperature and time parameters were as follows: an initial denaturing period of 4 min at 94°C, followed by 35 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec and a final extension period of 5 min at 72°C. PCR products were resolved on a 1% agarose gel, purified and sequenced.

DNA sequencing and analysis. RT-PCR products from rat cochlear cDNA were sequenced directly using ABI sequencer 3700 and AQP5 specific primers. Both, Sequencher and DNASTAR sequence analysis programs were used for sequence analysis and alignment.

Protein extraction and Western blot analysis. Tissue samples (lung, liver and cochleae) were harvested from adult Sprague Dawley rats and snap frozen in liquid nitrogen. The cochlear homogenate (whole cell extract) was prepared by grinding the micro-dissected cochleae (14 altogether) in cell lysis/gel loading buffer buffer (50 mM Tris–Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol) within a microcentrigue. The homogenate was passed through 26 gauge needle to shear genomic DNA. Unlike the cochleae, tissue samples of lung and brain were processed to yield membrane fractions. Briefly, the tissue samples were pulverized

and then resuspended in KB-buffer (30 mM Mannitol, 10 mM $CaCl_2$, 10 mM Tris pH 7.4, PMSF and Apoprotein). The resuspended samples were then homogenized by several strokes in a pre-cooled Dounce homogenizer. Large debris was removed by centrifugation at 3000 g for 5 min and 4°C. Then the supernatant was further cleared by a repetition of this centrifugation. The supernatant was then centrifuged at 35 K for 1 h at 14°C to precipitate the membrane fraction, subsequently resuspended in gel loading buffer. All samples, whole cell extract or membrane fraction, resuspended in gel loading buffer were heated for 15 min at 80°C and then resolved on 1.0 mm-thick 6% Tris-glycine minigel (NOVEX, CA) via electrophoreses at 125V for 90 minutes in Laemmli Running buffer. Electrophoretic transfer to nitrocellulose membrane (NOVEX) was then performed at 25 V for 120 minutes using Tris-glycine transfer buffer (Tris base 12 mM, glycine 96 mM, methanol 20%). The membrane was treated with 6% nonfat dry milk powder in 0.3% PBT at 4°C overnight to block nonspecific binding sites. The blot was then incubated with the affininty purified anti-AQP5 antibody (16) diluted 1/1,000 in 0.3% PBT at RT for 1 h. The blot was then washed in PBS followed by incubation with HRP-conjugated, goat anti-rabbit IgG (Amersham) for another one hour. After washing in PBS, the immunoblot was then developed using the ECL kit from Amersham.

Tissue processing and immunohistochemistry. Rats were anaesthetized as described, and the left ventricle was perfused through thoracotomy with saline followed by 500 ml of 4% paraformaldehyde in PBS. Lung, submandibular gland and temporal bones were harvested. The temporal bones were dissected to expose an intact cochlea whose stapes was removed to provide access to the oval window. The cochlea was then locally perfused with fixation solution through the round window and a small exit hole at the apex of the cochlea using a 20 gauge needle. All tissues were then immersed in fixation solution at 4°C overnight, dehydrated by a graded alcohol series and finally embedded in paraffin. Unlike the other tissues, cochleae were decalcified in 0.2 M EDTA for 2-3 weeks prior to susequent dehydration step. Paraffin embedded tissues were sectioned (6 μm) and mounted on pretreated glass slides (Superfrost, Fisher). Tissue sections were deparaffinized, rehydrated and then incubated with a blocking reagent, I-PBT (0.2% I-block (Tropix) and 0.05% Tween 20 in PBS). The blocked sections were incubated with aquaporin-5 antibody (1/100 dilution) in I-PBT for 1 h at RT, washed with PBS to remove unbound Ab, incubated with a biotin conjugated anti-rabbit IgG secondary antibody (Vector laboratories, 1/250 dilution) in I-PBT for 1 h at RT, washed and then incubated with alkaline phosphatase conjugated streptavidin 1/500 in I-PBT for 1 h at RT. The bound label was then detected using AP color substrte NBT-BCIP and coverslipped for microscopy. The specificity of immunoreactivity to the AQP5 Ab was assessed by incubating the primary antibody in presence of a competing peptide, 12 μg/ml, against which the antibody was raised.

RESULTS

RT-PCR and sequence analysis of AQP5 cDNA in rat cochlea. Several rat tissues including the cochlea were screened for expression of AQP5 transcript by RT-PCR. Using the primer pair A2B2 (see Materials and Methods), specific for rat AQP5 sequence, a 523 bp DNA was amplified from lung and cochlear cDNA (Fig. 1a); the fragment was not amplified from cDNAs of other tissues including brain and muscle. A similar tissue expression pattern was also found using primers A1B1 that were used to amplify the 5′-half of the AQP5 coding sequence (not shown). All cDNA samples tested for AQP5 expression were positive for CYPR expression (Fig. 1b). The nucleotide sequence of the amplified bands from the cochlea using AQP5-specific primers was identical to each the sequence reported previously for rat AQP5 cloned from the submandibular gland (14).

Western blot analysis. Whole cell extract of rat cochleae as well as membrane fractions of rat lung and liver were resolved via SDS-PAGE and characterized for AQP5 expression using anti-AQP5 Ab (Fig. 2). A predominant immunoreactive band of M_r 34 kDa accompanied by a minor band of M_r 34 kDa. The size of the lower band is identical to that of the immunoreactive band observed in the cochlear homogenate while the upper band, twice the size of the lower band may represent a dimer of the lower band. The anti-AQP5-immunoreactive 34 kDa band observed in both the lung and the 34 and 28 kDa bands in cochlear homogenate have been previously described; they correspond to the glycosylated and non-glycosylated forms of AQP5 (14, 16, 17). Membrane fraction from rat brain was negative for anti-AQP-5 immunoreactivity.

In situ localization of AQP-5 in the cochlea. Localization of AQP5 expression within the rat cochlea was determined via immunohistochemistry. Immunoreactivity of the cochlear sections to the AQP5 antibody was localized exclusively to the external sulcus cells (ES) and cells of the spiral prominence juxtaposed between the Claudius cells of the organ of Corti and the stria vascularis (Fig. 3a). The immunoreactivity was most intense in the ES cells and the spiral prominence of apical turn (Fig. 3c) and absent from the basal turn (Fig. 3d) of the cochlea. Anti-AQP5 immunoreactivity of the ES cells was eliminated in the presence of excess peptide antigen at a concentration of 10 μg/ml (Fig. 3b). The neuroepithelia and the supporting cells of the vestibular apparatus were negative for anti-AQP5 immunoreactivity (data not shown).

DISCUSSION

AQP5 has been previously identified in tissues and cell-types responsible for secretion of saliva, tears and pulmonary fluids. This study reports the identification and *in situ* localization of AQP5 in the cochlea that houses the auditory organ and its supporting structures. The cochlear AQP5 cDNA displayed complete sequence identity with the previously published *AQP5* cDNA cloned from the rat submandibular gland (14). Immunoblot analysis identified a predominant AQP5-immunoreactive protein species within the cochlear homogenate that has been previously described AQP5-positive tissue and cell types (14, 16, 17). The anti-AQP5 immunoreactive protein species was localized *in situ* to a single distinct region within the rat cochlear section that borders the endolymph. It is below stria

vascularis, vascular tissue considered to be responsible for generation of the endolymph and above the Claudius cells that are part of the supporting cells within the organ of Corti. The immunoreactive region for AQP5 is occupied by two distinct cell-types—the external sulcus (ES) cells and cells of the spiral prominence (18).

The ES cells are located between the spiral prominence and the Claudius cells. Apical surfaces of these cells interface with the endolymph. However, direct contact with the endolymph varies between the ES cells as the adjacent Claudius cells may cover their apical surfaces partly or almost entirely. The basal surface of these cells sends out long projections into the central region of the spiral ligament occupied by type II fibrocytes that express the ion transporting enzyme Na^+, K^+-ATPase (19, 20). Spiral prominence represents a vascularized region of the spiral ligament that bulges into the scala media just above ES cells and below the stria vascularis. It consists of a single row of cells (flat to cuboidal in shape) whose apical membranes interface with the endolymph.

Curiously, the immunoreactivity of the ES cells and spiral prominence against the anti AQP5 Ab was restricted to the upper turns of the rat cochlea and absent in the basal turn of the cochlea. This restricted pattern of anti-AQP5 immunoreactivity along the membranous labyrinth was competed by synthetic peptide antigen against which the Ab was raised. The competition studies support the operational specificity of the primary antibody to AQP5 and hence the tissue-specific and region-specific expression of AQP5 in the cochlea.

The sensory cells at the apical region of the cochlea where AQP5 expression is observed are optimally responsive or tuned to the low frequency tones while those at the basal region are tuned to high frequency. The sound frequencies processed by the sensory cells at the base and the apex of the cochlea differ in several orders of magnitude. These functional characteristics are reflected in number of structural differences in the hair cells and the surrounding support structures from the base to the apex of the cochlea and displayed in a gradient-like manner. The basillar membrane that partitions the cochlear duct and provides the base for the organ of Corti tapers in its stiffness but increases in its width as it extends from the base to the cochlea. The outer hair cells increase in their length from the base to apex while their stereocilia increase in length and decrease in numbers. These structural differences are also considered to reflect the distinct evolutionary origins of low and high frequency hearing (21, 22). Low frequency hearing is considered the primitive trait in the vertebrates while high frequency hearing is considered to have evolved later in evolution as it is found only among mammals. The physical parameters of the gradients contribute toward resolving a complex incoming sound stimulus into its constituent components or tones along the length of the cochlea.

The gradients in the physical structures along the length of the cochlea also raise questions concerning the underlying molecular constituents. Restricted expression of AQP5 to the apex of the cochlea, as demonstrated in this study, represents the first example of a molecular component that differs in its expression along the length of the cochlea. The restricted regional expression of AQP5 implies a potential functional role of AQP5 over a restricted frequency range, i.e., the low frequency hearing range.

The ES cells, spiral prominence and the stria vascularis form the lateral wall of the endolymphatic duct. The cells of this lateral wall have been extensively studied for their responses to variety of factors that may regulate their secretory and absorptive processes. One of the pathways through which these processes are induced is through the regulation of intra-cellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). Studies investigating mechanisms that regulate activity of AQP5 in the rat parotid gland have shown $[Ca^{2+}]_i$ dependent translocation of AQP5 between the intracellular membranes and the apical membranes of the paratoid gland. The increase in $[Ca^{2+}]_i$ was shown to be induced through the stimulation of cholinergic innervation to the parotid gland (23). The translocation of AQP5 between the intracellular and the apical membranes coincides temporally with the increase in $[Ca^{2+}]_i$ and the onset of salivary secretion in the parotid gland that is induced by cholinergic stimulation.

ATP, a modulator of variety of biological responses, is also considered to exert its effect through the regulation of $[Ca^{2+}]_i$. Characterization of the sensory and non-sensory cells of the organ of Corti, the auditory neuroepithelia, has demonstrated ATP mediated regulation of $[Ca^{2+}]_i$ in these cell types. Ikeda *et al.* (1995) characterized the lateral wall of the endolymphatic duct for its response to extra-cellular ATP. These studies revealed that the ES cells and the cells of the spiral prominence but not the stria vascularis were responsive to extra-cellular ATP induced increase in $[Ca^{2+}]_i$ (23)—the same cell-types that were found to be positive for AQP5 expression in this study. The ATP that may regulate $[Ca^{2+}]_i$ in the cell types lining the cochlear duct *in vivo* is speculated to be released as a humoral factor from surrounding tissues into the endolymph and not from efferent innervation of the lateral wall.

Two previous studies have also characterized the rat cochlea for presence of multiple members of the AQP gene family including AQP5. Takumi *et al.* (1998) raised antibodies for AQPs 1 to 5 and were able to immunolocalize AQP1 and 4 but not AQP2, 3 and 5 (24). Beitz *et al.* (1999) assayed for presence of the AQPs via RT-PCR analysis in cDNA prepared from microdissected cochlear tissues. Their RT-PCR assay identified AQP5 within the organ of Corti as well as Reissner's membrane (25). These regions of the cochlea were non-immunoreactive to the AQP5 Abs used in the current study. It is possible that AQP5 expression in

organ of Corti and Reissner's membrane is below the detection limit of immunolocalization. The inability of RT-PCR to detect AQP5 cDNA in the lateral wall of the cochlea may be a consequence of non-inclusion of the AQP5 positive cell types in the tissue sample that was assayed. Alternatively, the tissue sample assayed may have been derived from the basal turn of the cochlea and not from the apical turns. Discrepancy between the RT-PCR assay for AQP5 reported by Beitz *et al.* (1999) and immunohistochemical localization of AQP5 reported in the current study is also encountered for characterization of AQP4 in the inner ear. Tukami *et al.* (1988) immunolocalized AQP4 in the supporting cells of organ of Corti; however, Beitz *et al.* (1999) were not able to detect its expression via RT-PCR of RNA extracted from the organ of Corti.

Although the presence of AQP5 within the inner cochlea implies a physiological role in auditory function, no hereditary syndromic or nonsyndromic hearing loss loci map within the chromosomal region of the AQP5 gene. Naturally occurring mutations for AQP5 have not yet been detected in humans or in animal models. Hence, characterization of mouse knockout of AQP5 for auditory function will be critical for assessing physiological role of AQP5 in hearing.

In summary, we have cloned AQP5 cDNA from the rat cochlea, detected AQP5 polypeptide by immunoblot of rat cochlea and localized its expression *in situ* to the ES cellls and the cells of the spiral prominence. The restricted expression of AQP5 to the apical turns of the cochlea suggests a potential role in low frequency hearing.

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