Impaired Hearing in Mice Lacking Aquaporin-4 Water Channels*

Jiang Li and A. S. Verkman‡

From the Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521

A role for aquaporins (AQPs) in hearing has been suggested from the specific expression of aquaporins in inner ear and the need for precise volume regulation in epithelial cells involved in acoustic signal transduction. Using mice deficient in selected aquaporins as controls, we localized AQP1 in fibrocytes in the spiral ligament and AQP4 in supporting epithelial cells (Hensen’s, Claudius, and inner sulcus cells) in the organ of Corti. To determine whether aquaporins play a role in hearing, auditory brain stem response (ABR) thresholds were compared in wild-type mice and transgenic null mice lacking (individually) AQP1, AQP3, AQP4, and AQP5. In 4–5-week-old mice in a CD1 genetic background, ABR thresholds in response to a click stimulus were remarkably increased by >12 db in AQP4 null mice compared with wild-type mice (p < 0.001), whereas ABR thresholds were not affected by AQP1, AQP3, or AQP5 deletion. In a C57B16 background, nearly all AQP4 null mice were deaf, whereas ABRs could be elicited in wild-type controls. ABRs in AQP4 null CD1 mice measured in response to tone bursts (4–20 kHz) indicated a frequency-independent hearing deficit. Light microscopy showed no differences in cochlear morphology of wild-type versus AQP4 null mice. These results provide the first direct evidence that an aquaporin water channel plays a role in hearing. AQP4 may facilitate rapid osmotic equilibration in epithelial cells in the organ of Corti, which are subject to large K⁺ fluxes during mechanoelectric signal transduction.

The aquaporins (AQPs)1 are a family of small integral membrane proteins that function as water transporters. Phenotype analysis of mice lacking aquaporins has indicated that they play a physiological role in the kidney, central nervous system, gastrointestinal system, and exocrine glands (reviewed in Ref. 1). Mice lacking AQP1, AQP2, or AQP3 manifest nephrogenic diabetes insipidus with defective urinary concentrating ability (2–4). Mice lacking AQP1, AQP2, or AQP3 manifest nephrogenic diabetes insipidus (2–4), mice lacking AQP4 in gastric parietal cells and skeletal muscle have unimpaired stomach acid secretion (8) and skeletal muscle function (9). The data from aquaporin null mice suggest that aquaporins are functionally important in tissues carrying out rapid near isosmolar fluid transport or passive water transport driven by osmotic gradients (1).

Several aquaporins have been localized in the mammalian inner ear and have been proposed to play a role in hearing. Reverse transcriptase polymerase chain reaction analysis of dissected rat inner ear showed diffuse AQP1 transcript expression, specific expression of AQP2, AQP3, and AQP4 in the endolymphatic sac, and expression of AQP5 in the organ of Corti and Reissner’s membrane (10). AQP1 was immunolocalized in guinea pig inner ear in fibrocytes near the bone and lining the endolymphatic duct and sac (11). Takumi et al. (12) found only AQP1 and AQP4 in rat inner ear by immunostaining, with AQP4 localized to the basolateral membrane of Hensen’s cells and basal plasma membranes of Claudius cells and inner sulcus cells. Similar findings were reported by Minami et al. (13). In one study, AQP5 was immunolocalized in cells lining the lateral wall of rat cochlear duct, including the external sulcus and spiral prominence (14). Although there is some disagreement among these expression studies, it appears that several aquaporins are expressed in the inner ear and thus might participate directly or indirectly in hearing. Recently, Belyantseva et al. (15) reported that water permeability in isolated rat cochlear outer hair cells increased at 8–12 days after birth, corresponding in time to the onset of hearing. Taken together with evidence that precise cochlear cell volume regulation is critical to mechanoelectric signal transduction (16), it has been suggested without direct evidence that aquaporins play an important role in hearing. However, hearing impairment in humans is not associated with nephrogenic diabetes insipidus caused by mutations in AQP2 or in reportedly asymptomatic humans lacking AQP1 (17).

The purpose of this study was to investigate the functional role of aquaporins in hearing. The strategy was to compare auditory brainstem response (ABR) signals in mice lacking each of the inner ear aquaporins. We found remarkable hearing impairment in mice lacking AQP4 without anatomical abnormalities. The data provide the first direct evidence for a role of an aquaporin in hearing, raising the possibility that AQP4 may be involved in some forms of hearing impairment and that modulation of AQP4 function in inner ear may be of therapeutic value.

MATERIALS AND METHODS

Transgenic Mice—Transgenic knockout mice deficient in AQP1, AQP3, AQP4, and AQP5 (individually) in a CD1 genetic background were generated by targeted gene disruption as described previously (2, 3, 6, 18). Measurements were done in litter-matched wild-type and knockout mice produced by intercrossing of heterozygous mice. For

* This study was supported by National Institutes of Health Grants HL59198, DK35124, HL60288, and DK43840 and Grant R613 from the National Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Cardiovascular Research Institute, 1246 Health Sciences East Tower, Box 0521, University of California, San Francisco, San Francisco, CA 94143-0521. Tel.: 415-476-8530; Fax: 415-665-3847; E-mail: verkman@itsa.ucsf.edu.

1 The abbreviations used are: AQP, aquaporin; ABR, auditory brain stem response; PBS, phosphate-buffered saline.
experiments in an inbred strain, the AQP4 null genotype was transferred to the C57/bl6 background by >8 back-crosses. For ABR measurements the investigators were blinded to genotype information until completion of the analysis. Protocols were approved by the University of California, San Francisco Committee on Animal Research.

**ABR Measurements**—ABR measurements were performed using a Biopac MP100 work station equipped with differential amplifier (ERS100B) and stimulator (STM100A) modules. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (14 mg/kg) by intraperitoneal injection. Rectal temperature was monitored continuously using a digital thermistor and maintained at 36–38 °C using a heating block. Mice were placed in a grounded Faraday cage sound isolation box. The three recording electrodes and speaker input cables entered a small hole in the box.

Biaural sound stimuli were produced by a broad band speaker (Realistic, Model 40-1310B) positioned 30 cm from the mice. Click stimuli were generated by a square pulse of 0.1-ms duration and specified amplitude. Tone stimuli were generated using a frequency synthesizer and home-built waveform modulator to give a trapezoidal waveform with 1-ms rise/fall times and 2-ms flat segment. Sound intensity calibrations were done using a model C550H measurement microphone (Josephon Engineering) positioned at the location of the mouse head. For recording, subdermal stainless steel needle electrodes were placed at the vertex and ventrolateral to the left and right ears. ABR waveforms were recorded in 10-db intervals down from a maximum amplitude of 90 db (for click stimuli) until no waveform could be visualized. Waveforms were stored for off-line analysis.

**Immunocytochemistry and Histological Analysis**—Samples were fixed by intracardiac perfusion with 4% paraformaldehyde in PBS (pH 7.4). The temporal bone was removed, and the cochlea was post-fixed overnight in the same fixative solution. After decalcification, the cochlea was dehydrated and embedded in Tissue-Tek OCT compound for cryostat sections and in glycol methacrylate for plastic sections. For histological examination, the cochlea was infiltrated with JB-4 monomer (Polyscience Inc.), embedded under vacuum at room temperature, sectioned on a microtome (Sorvall), and stained with toluidine blue. For immunocytochemistry, 3–4-μm thick cryostat sections were incubated for 30 min with PBS containing 1% bovine serum albumin and then with affinity-purified aquaporin antibodies (dilution 1:100–1:1500) for 2 h at 3°C in PBS containing 1% bovine serum albumin. Slides were rinsed with 2.7% NaCl and then with PBS and incubated with a secondary Cy3-conjugated sheep anti-rabbit P(ab)2 fragment (1:200) for visualization by fluorescence microscopy. 

**Data Analysis**—Data are reported as the mean ± S.E. with p values determined by analysis of variance.

**RESULTS**

Aquaporin localization in the organ of Corti in the inner ears of four sets of mice was done by immunostaining using specific antibodies (Fig. 1). The AQP1 antibody strongly labeled non-epithelial cells (fibrocytes) in the spiral ligament of wild-type mice (top left) with no labeling in AQP1 null mice (top right). The AQP4 antibody labeled supporting Hensen’s cells, inner sulcus cells, and Claudius cells in wild-type mice (middle left) but not in AQP4 null mice (middle right). No immunostaining with AQP5 antibody was found (bottom left and right), despite strong label of other tissues known to express AQP5 such as salivary gland (inset). Immunostaining of AQP2 and AQP3 was negative, with strongly positive controls (mouse kidney, not shown).

Hearing was evaluated functionally in wild-type and aquaporin null mice of age 4–5 weeks by ABR analysis. Fig. 2 shows representative ABR waveforms in response to click stimuli of different intensities. As reported in other ABR studies in mice (19), at least four distinct peaks were identified corresponding to cochlear nerve activity (wave I) and downstream neural activity (waves II–IV). Decreasing click intensities resulted in a decrease in wave amplitudes. As generally defined, the ABR threshold was identified in each series of ABR waveforms as the lowest click intensity that produced at least two clearly visible waves. ABR thresholds for the data in Fig. 2 were 40 db (wild-type mouse) and 50 db (AQP4 null mouse). Control studies indicated that ABR thresholds were very reproducible in the same mice measured on different days, with identical ABR thresholds in ~80% of mice and 5 db changes in most remaining mice.

Fig. 3 summarizes ABR thresholds measured in a large series of wild-type and aquaporin null mice. Although typical variability was found in different mice in the outbred CD1 genetic background, there was a significantly increased ABR threshold in AQP4 null mice by >12 db (p < 0.001). ABR thresholds in mice lacking AQP1, AQP3, or AQP5 did not differ significantly from that in wild-type mice. Further studies were done in C57/bl6 inbred mice into which the AQP4 null genotype was transferred. Fig. 3 (right) shows that most AQP4 null mice were deaf, whereas ABR waveforms could be elicited in...
matched wild-type mice. The results indicated remarkably impaired hearing in AQP4 null mice.

ABR waveforms from wild-type and AQP4 null mice in the CD1 background were further analyzed. Fig. 4A shows wave I amplitudes measured at 70, 60, and 50 db click intensities. At each click intensity, the amplitudes of wave I were significantly greater for wild-type than for AQP4 null mice. Fig. 4B summarizes amplitude ratios of wave I/wave II and wave I/wave III for each mouse. There was no significant different in amplitude ratios in wild-type versus AQP4 null mice. Together, these results indicate that the hearing impairment arises from defective cochlear rather than downstream neural function.

Tone ABR analysis was done to determine whether the hearing impairment in AQP4 null mice was frequency-dependent. Modulated tone bursts were created using a custom-built frequency synthesizer-modulator. Fig. 5A shows time-domain waveforms along with frequency spectra deduced by Fourier analysis of single clicks and tone bursts. Fig. 5B shows representative ABR waveforms in response to tone bursts. Qualitatively, ABR thresholds were increased in AQP4 null mice at all frequencies. Fig. 6 summarizes click and tone ABR thresholds for a series of wild-type and AQP4 null mice, indicating that the hearing impairment in AQP4 null mice is not frequency-specific.

Morphological examination of plastic sections of inner ear was done to determine whether anatomical differences could account for the impaired hearing in AQP4 null mice. Fig. 7 (top) shows representative sections from two wild-type and two AQP4 null mice. The thin (2 µm) plastic sections clearly show an intact organ of Corti with well demarcated hair cells and supportive cells. No morphological differences were apparent in sections of inner ear evaluated blind from four wild-type and four AQP4 null mice. Fig. 7 (bottom) shows a schematic of the mouse cochlea based on the immunocytochemistry and histology studies (see “Discussion”).

**DISCUSSION**

The principal goal of this study was to determine whether aquaporins play a functional role in hearing. Because there was disagreement in the literature about aquaporin expression patterns in mammalian inner ear, and no information was available to our knowledge on mouse inner ear, we determined by immunocytochemistry the expression pattern of aquaporins in mouse inner ear. Knockout mice lacking individual aquaporins served as controls. We found AQP1 protein in non-epithelial cells in spiral ligament and AQP4 in the basolateral plasma membranes of Hensen’s cells and inner sulcus cells and the basal plasma membrane of Claudius cells in agreement with previous reports in guinea pig and/or rat. We did not find specific immunostaining of AQP2, AQP3, or AQP5, despite appropriate positive controls using mouse tissues known to express these aquaporins. Nevertheless, recognizing the limitations of antibody detection of aquaporins, ABR analysis was done on wild-type mice and knockout mice lacking AQP1, AQP3, AQP4, and AQP5. Although an AQP2 knock-in mouse model of autosomal recessive nephrogenic diabetes insipidus was recently created (4), these mice were not suitable for ABR analysis because they generally did not survive beyond the first week of life. We found remarkable hearing impairment in AQP4 null mice, with no significant effect of deletion of AQP1, AQP3, or AQP5. The hearing-impaired AQP4 null mice did not show abnormalities in cochlear morphology at the light microscopic level. These results suggest that AQP4-mediated water transport in supportive epithelial cells in the organ of Corti is required for normal hearing.

ABR analysis in mice is an established approach to detect hearing impairment resulting from a variety of genetic and acquired diseases (20–23). The measurements here were done...
in wild-type (open circles) and AQP4 null (closed circles) CD1 mice of age 4–5 weeks. Averaged data (mean ± S.E.) are shown for click and tone stimuli. *, indicates significant difference compared with wild-type mice: *, p < 0.001; ** p < 0.05.

FIG. 5. Frequency-dependent hearing measured by tone ABR analysis. A, frequency spectra of click and tone pips detected using a microphone and Fourier analysis. Time-domain speaker waveforms are shown as insets. B, representative ABR waveforms measured in wild-type and AQP4 null CD1 mice in response to tone stimuli of indicated frequencies and intensities.
astroglia is proposed to facilitate K⁺ flux in supporting cells may be a general paradigm in the physiology of neuroexcitable tissues. In the central nervous system, AQP4 in astroglia is proposed to facilitate K⁺ fluxes associated with adjacent neurons (38–40), and in the eye AQP4 in retinal Muller cells is proposed to facilitate K⁺ fluxes associated with adjacent bipolar cells (41). A close molecular association of AQP4 in orthogonal arrays of particles with K⁺ channels has been proposed (42). The challenge will be to establish the cellular and molecular mechanisms by which AQP4 facilitates K⁺ and water movement in excitatory tissues.

Acknowledgment—We thank Liman Qian for transgenic mouse breeding and genotype analysis.

REFERENCES

1. Verkman, A. S., Yang, B., Song, Y., Manley, G. T., and Ma, T. (2000) Exp. Physiol. 85, suppl. 233S–241S
2. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1998) J. Biol. Chem. 273, 4296–4299
3. Ma, T., Song, Y., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4386–4391
4. Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (2001) J. Biol. Chem. 276, 2770–2779
5. Manley, G. T., Fujimura, M., Ma, T., Noshita, N., Filiz, F., Bollen, A., Chan, P., and Verkman, A. S. (2000) Nat. Med. 6, 159–163
6. Ma, T., Song, Y., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1999) J. Biol. Chem. 274, 20071–20074
7. Deen, P. M., Verkijk, M. A., Knoers, N. V., Wieringa, B., Monnens, L. A., Van Os, C. H., and Van Oost, B. A. (1994) Science 264, 92–95
8. Wang, K. S., Komar, A. R., Ma, T., Filiz, F., McLeroy, J., Hoda, K., Verkman, A. S., and Bastidas, J. A. (2000) Am. J. Physiol. 279, G448–G453
9. Yang, B., Verbavatz, J. M., Song, Y., Manley, G. T., Vetrivel, L., Kas, M. M., Ma, T., and Verkman, A. S. (2000) Am. J. Physiol. 278, C1108–C1115
10. Beitz, E., Kumagame, H., Krippeit-Drewes, P., Ruppersberg, J. P., and Schulz, J. E. (1999) Hear. Res. 132, 76–84
11. Stankovic, K. M., Adams, J. C., and Brown, D. (1995) Am. J. Physiol. 269, C1450–C1456
12. Takumi, Y., Nagelhus, E. A., Eide, J., Matsushita, A., Usami, S., Shinkawa, H., Nielsen, S., and Ottersen, O. P. (1998) Eur. J. Neurosci. 10, 3584–3585
13. Minami, Y., Shimagawa, H., Matsuura, H., Matsuoka, T., and Tsuchiya, M. Acta Otolaryngol. Suppl. (Stockh.) 533, 19–21
14. Mhatre, A. N., Steinbach, S., Hribar, K., Hoque, A. T., and Lalwani, A. K. (1999) Biochim. Biophys. Res. Commun. 264, 157–162
15. Belyantseva, I. A., Frenkelov, G. I., Wade, J. B., Mammano, F., and Kachar, B. (2000) J. Neurosci. 20, 8966–9003
16. Patuzzi, R. (1996) The Cochlea (Dallas, P., Popper, A. N., and Fay, R. R., eds) pp. 186–257, Springer-Verlag, New York
17. Preston, G. M., Smith, B. L., Zeidell, M. L., Moulds, J. J., and Agre, P. (1994) Science 265, 1585–1587
18. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1997) J. Clin. Invest. 100, 957–962
19. Zheng, Q. Y., Johnson, K. R., and Erway, L. C. (1999) Hear. Res. 130, 94–107
20. Erway, L. C., Shiu, Y. W., Davis, R. R., and Krieg, E. F. (1996) Hear. Res. 93, 181–187
21. Henry, R. K., McGinn, M. D., Carter, L. A., and Savoska, E. A. (1992) Audiology 31, 190–195
22. Johnson, K. R., Erway, L. C., Cook, S. A., Willott, J. F., and Zheng, Q. Y. (1997) Hear. Res. 114, 83–92
23. Willott, J. F., Erway, L. C., Archer, J. R., and Harrison, D. E. (1995) Hear. Res. 88, 143–155
24. Markland, O. N. (1994) J. Clin. Neurophysiol. 11, 319–324
25. Muller, A. R. (1994) J. Clin. Neurophysiol. 11, 284–298
26. Yang, B., and Verkman, A. S. (1997) J. Biol. Chem. 272, 16140–16146
27. Hasegawa, H., Ma, T., Skach, W., Matthay, M., and Verkman, A. S. (1994) J. Biol. Chem. 269, 5497–5500
28. Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 13052–13056
29. Misaka, T., Abe, K., Iwabuchi, K., Kusakabe, Y., Ichinose, M., Miki, K., Emori, Y., and Arimura, S. (1996) FEBS Lett. 381, 208–212
30. Chou, C. L., Ma, T., Yang, B., Knepper, M. A., and Verkman, A. S. (1998) Am. J. Physiol. 274, C549–C554
31. Frigeri, A., Gropper, M., Turk, C. W., and Verkman, A. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4328–4331
32. Nagelhus, E. A., Veruki, M. L., Torp, R., Haug, F. M., Laake, J. H., Nielsen, S., Agre, P., and Ottersen, O. P. (1998) J. Neurosci. 18, 2506–2519
33. Yang, B., Brown, D., and Verkman, A. S. (1996) J. Biol. Chem. 271, 4577–4580
34. Verbavatz, J. M., Ma, T., Gobin, R., and Verkman, A. S. (1997) J. Cell Sci. 110, 2855–2860
35. Sakaguchi, N., Crouch, J. Y., Lytle, C., and Schulte, B. A. (1998) Hear. Res. 118, 114–122
36. Kubisch, C., Schroeder, B. C., Friedrich, T., Lotjohann, B., El-Amraoui, A., Marin, S., Petit, C., and Jentsch, T. (1999) Cell 96, 437–446
37. Steel, K. P. (1999) Science 285, 1363–1364
38. Trayanova, N. S., and Dingledine, R. (1989) J. Neurophysiol. 61, 927–938
39. Randam, B. R., and Orkand, R. K. (1996) Trends Neurosci. 19, 352–358
40. Nielsen, S., Nagelhus, E. A., Amiry-Moghaddam, M., Bourque, C. W., Agre, P., and Ottersen, O. P. (1997) J. Neurosci. 17, 171–180
41. Nagelhus, E. A., Horis, Y., Inazoshi, A., Fujita, A., Haug, F. M., Nielsen, S., Kurachi, Y., and Ottersen, O. P. (1999) Glia 26, 47–54
42. Rash, J. E., Tatumura, T., Hudson, C. S., Agre, P., and Nielsen, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11981–11986
43. Erway, L. C., Willott, J. F., Archer, J. R., and Harrison, D. E. (1993) Hear. Res. 65, 125–132
