Immunolocalization of Voltage-Gated Potassium Channel Kv3.4 Subunit in the Cochlea

Kotaro Ishimaru[1], Toshihiko Kikuchi[1,2], Yuka Miyabe[1], Eigo So[1], Naohiro Yoshida[2], Toshimitsu Kobayashi[2], Haruo Takahashi[1]

1) Division of Otorhinolaryngology Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

2) Department of Otorhinolaryngology-Head and Neck Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Immunohistochemical localization of voltage-gated potassium channel Kv3.4 subunit was studied in the guinea-pig cochlea. Kv3.4-like immunoreactivity was present in the type I and suprastrial fibrocytes of the spiral ligament and the basal cells of the stria vascularis. Immunostaining was also found in the type II-IV fibrocytes of the spiral ligament and the connective tissue cells of the spiral limbus. In the organ of Corti, Kv3.4-like immunoreactivity was found in the sensory cells and the neighboring supporting cells. The root cells and interdental cells were positively immunostained. Immunoreactivity was also found in the endothelial cells of the blood vessels in the cochlear modiolus. These results suggest that Kv3.4 may participate in the potassium ion recycling mechanism in the cochlea.

ACTA MEDICA NAGASAKIENSIA 48 : 153 — 158, 2003

Introduction

The apical plasma membranes of cochlear sensory cells face endolymph, which has a characteristic ionic composition of high potassium and low sodium concentration and the endocochlear potential (EP) of 80 - 100 mV[1]. The acoustically evoked receptor potential is generated by the influx of potassium ions from the endolymph into the sensory cells. These K$^+$ ions are then released to the extracellular space and from here are recycled back to endolymph.

In 1995, Kikuchi et al.[2] revealed the immunohistochemical

Address Correspondence: Kotaro Ishimaru, M.D.
Department of Otolaryngology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
TEL: +81-95-849-3023, FAX: +81-95-849-7352
E-mail: kotaro@hakujyujikai.or.jp

localization of connexin 26, a gap junction protein, in the mammalian inner ear, and clearly demonstrated the existence of the two independent gap junction systems in the cochlea. The first system, *the epithelial cell gap junction system*, is mainly composed of supporting cells of the organ of Corti, and also includes root cells and interdental cells. The second system, *the connective tissue cell gap junction system*, consists of basal and intermediate cells of the stria vascularis, fibrocytes in the spiral ligament, mesenchymal cells lining the scala vestibuli and the connective tissue cells in the spiral limbus. These two networks of gap junctions are most likely the pathway for recirculation of cochlear K$^+$ ions[2-5]. Recent molecular genetics studies[6,7] have shown that mutations of connexin 26 gene can cause non-syndromic deafness, a finding that strongly support the functional significance of gap junctions in the ion recycling mechanism in the inner ear.

In the mammalian cochlea, the movement of potassium ions is dependent upon the activities of Na, K-ATPase[3,8], Na-K-Cl cotransporter (NKCC1)[8] and various types of potassium channels[9-15].

Voltage-gated potassium (Kv) channels form the extensive and diversified class of ion channels[16,17], and are present in both excitable and nonexcitable cells. In the excitable cells, Kv channels are involved in the regulation of the resting membrane potential and the control of the shape and frequency of action potentials. They play important roles in muscle contraction, cardiac pacemaking and hormone secretion, cell proliferation, cell volume regulation and lymphocyte differentiation.

In 2001, So et al.[9] reported that the voltage-gated potassium channel Kv3.1b subunit is widely expressed in the type I, type III, type IV and suprastrial fibrocytes of the guinea pig cochlear lateral wall, and suggested that Kv3.1b may play some important roles

in regulating the potassium ion recycling mechanism via gap junctions in the mammalian inner ear.

The voltage-gated potassium channel Kv3.4 forms an A-type inactivating current[16, 17]. Recent studies have shown that Kv3.4 is expressed in the dentate gyrus of the hippocampus, the pontine nuclei of the brainstem, cerebellum[17, 18]. Kv3.4 is also known to be expressed in the smooth muscle cells of the tail artery[19].

In the present investigation, the immunohistochemical localization of voltage-gated potassium channel Kv3.4 subunit was studied in the guinea pig inner ear. The possible role of Kv3.4 in the inner ear function is also discussed.

MATERIALS AND METHODS

Under the deep anesthesia with an intraperitoneal injection of sodium pentobarbital (28 mg/kg) and an inhalation of diethyl ether, six guinea pigs, 300-380 g in body weight, were transcardially perfused with 0.01 M phosphate buffered saline (PBS), pH 7.2, followed by 10% formalin in PBS. The middle ear cavity was opened rapidly, the stapes was removed, the round window membrane was pierced, and the perilymphatic space was gently perfused with 10% formalin containing 1% acetic acid in PBS. The temporal bones were rapidly removed and immersed in the fixative. After fixation for 2 hours at room temperature, the perilymphatic cavity was flushed with PBS. The temporal bones were decalcified in 0.12 M ethylenediaminetetraacetic acid (EDTA), pH 7.0, for 3 - 4 weeks at room temperature. They were dehydrated with a graded series of ethanol solutions, cleared in xylenes, and embedded in paraffin. Sections were cut continually at 6 mm and mounted on slides coated with poly-L-lysine and every 20th section was stained with hematoxylin and eosin.

The mounted sections were deparaffinized, rehydrated, and exposed to 5% normal goat serum in PBS for 1 hour. They were incubated at room temperature overnight with a dilution of 1:200 - 1:400 of rabbit anti-Kv3.4 polyclonal antibody (Alomone labs, Jerusalem, Israel) in 1% bovine serum albumin in PBS at room temperature overnight. This antibody was raised against highly purified peptide EAGDDERELALQRLGPHEG(C), corresponding to residues 177-195 of rat or 176-194 of human Kv3.4, with additional C-terminal cysteine. After washing with PBS, the sections were flooded for 30 minutes with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Envision+, DakoCytomation California Inc., Carpinteria, CA, USA), and rinsed in PBS. The immunohistochemical reaction was visualized by development for 10 minutes in 3,3'-diaminobenzidine

tetrahydrochloride (DAB)-H_2O_2 substrate medium prior to dehydration and cover slipping.

Cytochemical controls entailed replacing the primary antiserum with a similar dilution of non-immune rabbit serum. The primary antibody was preincubated with the Kv3.4 control peptide as a further specificity control.

RESULTS

As illustrated in Figure 1, an intense and highly specific voltage-gated potassium channel Kv3.4 subunit-like immunoreactivity was present in the
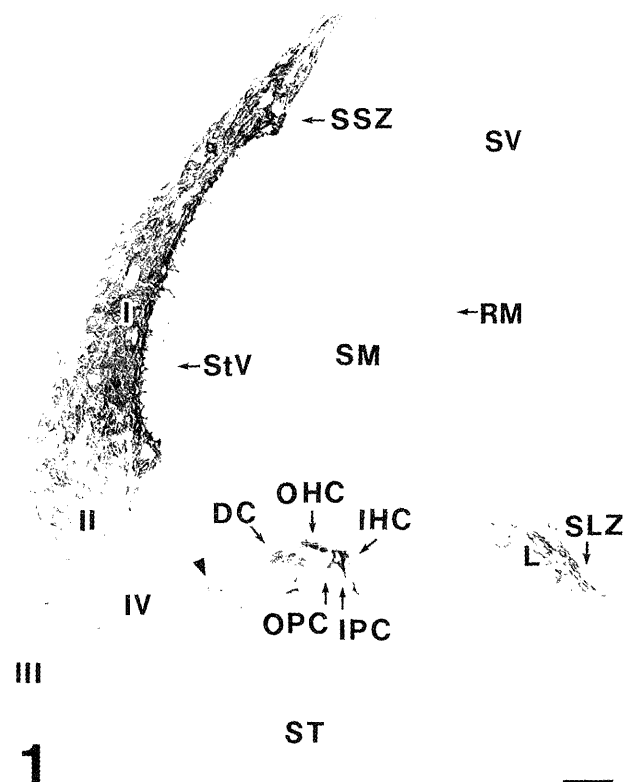

Figure 1. Paraffin section of guinea pig cochlea immunostained with anti-Kv3.4 antibody. In the cochlear lateral wall, intense Kv3.4 subunit-like immunoreactivity is observed in type I fibrocytes (I), fibrocytes in the suprastrial zone (SSZ) and the basal cells in the stria vascularis. Weak Kv3.4-like immunoreactivity is observed in the type II (II), type III (III) and type IV (IV) fibrocytes in the spiral ligament. Intense immunostaining is also found in the mesenchymal dark cells in the supralimbal zone (SLZ) and nearby fibrocytes in the spiral limbus. Immunostaining is also found in the outer hair cells (OHC) and inner hair cells (IHC) of the organ of Corti. Cochlear supporting cells, including the Boettcher's cells (arrowhead), Deiters' cells (DC) and outer pillar cells (OPC) and inner pillar cells (IPC), are also positively immunostained. L, spiral limbus; RM, Reissner's membrane; SM, scala media; ST, scala tympani; StV, stria vascularis; SV, scala vestibuli. Bar = 50mm
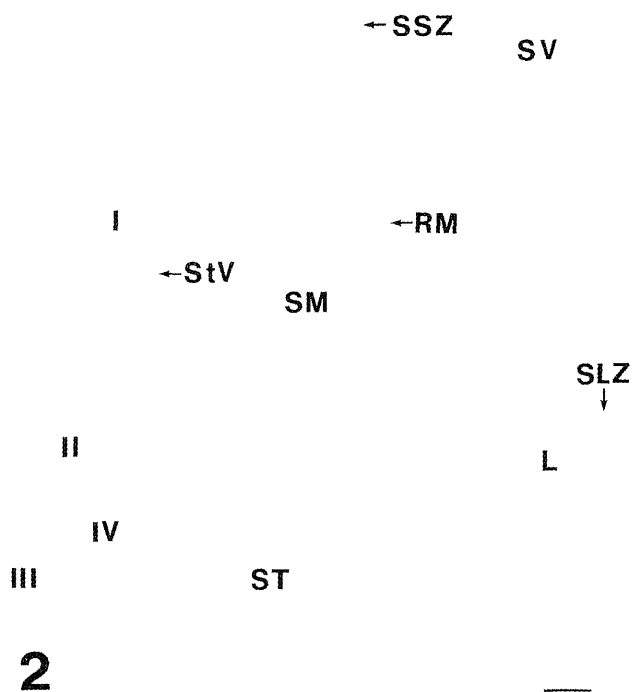
←SSZ

SV

I   ←RM

←StV

SM

SLZ
↓

II   L

IV

III   ST

2   ___

Figure 2. Section from the region similar to that depicted in Fig. 2. Application of the primary antibody, which was preabsorbed with Kv3.4 control peptides, in the first step of immunostaining sequence resulted in no specific staining. I, type I fibrocyte area; II, type II fibrocyte area; III, type III fibrocyte area; IV, type IV fibrocyte area; L, spiral limbus; RM, Reissner's membrane; SLZ, supralimbal zone; SM, scala media; SSZ, suprastrial zone; ST, scala tympani; StV, stria vascularis; SV, scala vestibuli. Bar = 50mm.

guinea pig cochlea. In contrast, no positive immunostaining was observed in the control sections where the primary antibody was replaced by the non-immune serum or the primary antiserum, which was preabsorbed with the Kv3.4 control peptides (Fig. 2).

In the cochlear lateral wall, intense Kv3.4-like immunoreactivity was observed in the basal cells of the stria vascularis (Fig. 1, Fig. 3a), the type I fibrocytes, which are distributed lateral to the stria vascularis, and in the fibrocytes in the suprastrial zone (Fig. 1). Weak Kv3.4-like immunoreactivity was observed in the type II, type III and type IV fibrocytes in the spiral ligament (Fig. 1). Intense immunostaining was also found in the supralimbal mesenchymal dark cells and nearby fibrocytes in the spiral limbus (Fig. 1, Fig. 3d).

In the organ of Corti, intense Kv3.4-like immunoreactivity was found in the stereocilia, apical plasma membrane and the infracuticular zone of the outer and inner hair cells (Fig. 1, Fig. 3c). Cochlear supporting

cells, including the Boettcher's cells, Deiters' cells and outer and inner pillar cells, were also positively immunostained (Fig. 1). Reaction product was present in the root cells in the lower part of the spiral ligament (Fig. 3b) and the interdental cells in the spiral limbus (Fig. 3d).

The endothelial cells of the blood vessels in the modiolus of the cochlea were also positively immunostained (Fig.3e). In contrast, no reaction product was detected in the capillaries in the stria vascularis (Figs. 3a).

DISCUSSION

In the present study, we have shown that intense voltage-gated Kv3.4 subunit-like immunoreactivity is present in the connective tissue cells, including the type I and suprastrial fibrocytes in the spiral ligament and the basal cells of the stria vascularis. Intense Kv3.4-like immunostaining was also found in the epithelial cells, including the sensory hair cells and the neighboring supporting cells of the organ of Corti. In the mammalian cochlea, the movement of potassium ions can be controlled by the various types of potassium channel proteins, including Kv3.1b[9], KCNQ1 (or KvLQT1)[10], IsK[11], KCNQ4[12], Kir4.1[13], ether a go-go (eag)[14], and maxi-K (or BK) channel[15].

In 2001, So et al.[9] have shown voltage-gated potassium channel Kv3.1b subunit-like immunoreactivity in the type I fibrocytes, type III fibrocytes, type IV fibrocytes and suprastrial fibrocytes in the guinea pig cochlear lateral wall, and suggested that Kv3.1b in the fibrocytes of the cochlear lateral wall may play an important role in regulating the potassium ion recycling mechanism via gap junctions in the inner ear.

Lecain et al.[14] have shown that the eag mRNA was expressed in the type I, type III, type IV and suprastrial fibrocytes of the cochlear lateral wall and in some epithelial cells of the organ of Corti.

Liang et al.[15] have shown that the voltage- and Ca²⁺-dependent maxi-K channels in the type I fibrocytes of the gerbil cochlear lateral wall.

It is interesting to note that the basic distribution pattern of Kv3.4 in the cochlear lateral wall is comparable to that of Kv3.1b, eag and maxi-K channels in the cochlear lateral wall. The co-expression of these four different types of voltage-gated potassium channel proteins in the fibrocytes in the cochlear lateral wall strongly suggests that the voltage-gated potassium channels in the connective tissue cells of the spiral ligament may be essential in the normal functioning of the mammalian cochlea.
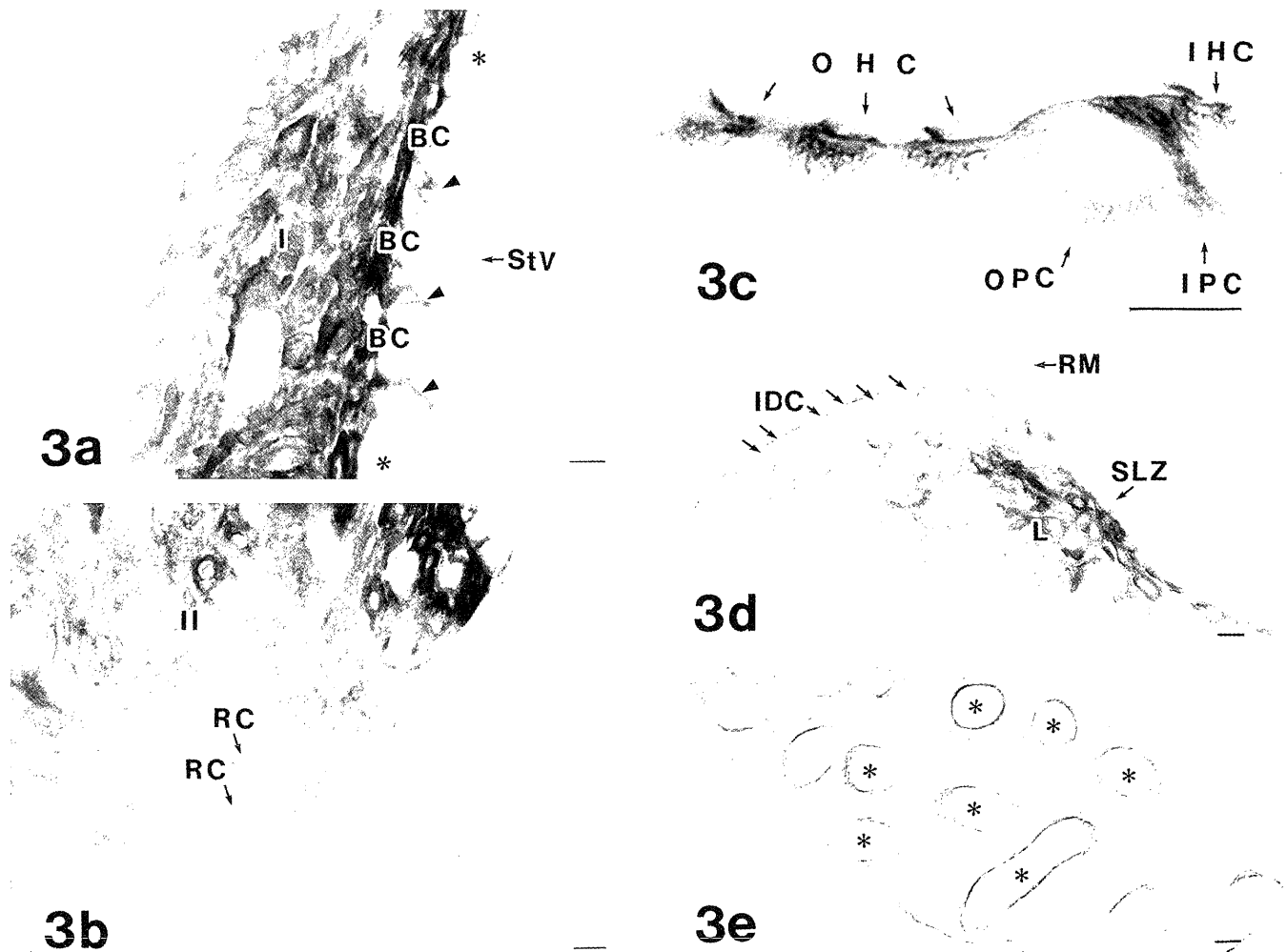
Figure 3. (a, b) A higher magnification of the cochlear lateral wall, immunostained with anti-Kv3.4 antibody. (a) The stria vascularis and the nearby type I fibrocyte area of the spiral ligament. Intense immunoreactivity is found in the basal cells (BC) in the stria vascularis and type I fibrocytes (I) in the spiral ligament. No reaction product is detected in the other components in the stria vascularis (StV), including the marginal cells, intermediate cells and the endothelial cells of the intrastrial capillaries (asterisks). Arrowheads, cytoplasmic processes of the basal cells of the stria vascularis. Bar=10mm. (b) The lower part of the spiral ligament. The root cells (RC) are positively immunostained. II, type II fibrocytes. Bar=10mm.

(c) A higher magnification of the cochlea hair cells immunostained with anti-Kv3.4 antibody. Intense Kv3.4-like immunoreactivity is found in the stereocilia, the apical plasma membrane and the subcuticular region of the outer hair cells (OHC) and inner hair cells (IHC). IPC, inner pillar cells; OPC, outer pillar cells. Bar=10mm.

(d) A higher magnification of the spiral limbus immunostained with anti-Kv3.4 antibody. Intense immunostaining is observed in the mesenchymal dark cells of the supralimbal zone (SLZ) and nearby fibrocytes in the spiral limbus. Immunostaining is also observed in the interdental cells (IDC). L, spiral limbus; RM, Reissner's membrane. Bar=10mm.

(e) A higher magnification of the blood vessels (asterisks) in the modiolus of the cochlea immunostained with anti-Kv3.4 antibody. The endothelial cells of the blood vessels in the modiolus of the cochlea are also positively immunostained. Bar=10mm.

Ionic exchanges via gap junctions are known to be affected by transjunctional voltage *(Vj)*[20]. Recently, membrane voltage *(Vm or Vi-o)*-sensitive gating has been documented in vertebrate gap junctions[21, 22]. In the mammalian inner ear, Zhao and Santos-Sacchi[22] examined the voltage-dependence of gap junctional conductance in cochlear supporting cells, and have found asymmetric voltage-dependencies for both *Vj* and *Vm*.

These data suggest that the conductance of gap junctions in the inner ear can be controlled by the intracellular electrical potentials, and also indicate that the movement of potassium ions via gap junctions in the inner ear can be controlled by the electrical potentials of the constituent cells of the gap junction systems in the inner ear. The control of the electrical potential in the fibrocytes of the cochlear lateral wall and in the

supporting cells of the organ of Corti by Kv3.4 may play a fundamental role in regulating the K$^+$ ion recycling mechanism in the inner ear.

Recent studies suggested that maxi-K channels[15], and Kv3.1b[9], might serve to regulate the K$^+$ concentration in the type I fibrocytes of the spiral ligament during its intracellular transit via gap junctions from type II fibrocytes of the spiral ligament to basal cells of the stria vascularis. It is also probable that Kv3.4 may play a comparable role in the potassium ion recycling mechanism via gap junctions in the cochlear lateral wall and in the organ of Corti.

In the organ of Corti, we have also shown that Kv3.4, which forms an A-type potassium current, is strongly expressed in the stereocilia, the apical plasma membrane and the infracuticular zone of the cochlear sensory cells. An electrophysiological study[23] has clearly shown the existence of A-type current in the sensory hair cells of the mammalian inner ear, and suggested that A-type current may play a role in stabilizing membrane potential in these sensory cells. It is also suggested that the canalicular reticulum in the infracuticular zone of the cochlear sensory cells provides a possible structural basis for sequestering the apical K$^+$ influx and for directing its diffusion to the site of efflux across the lateral plasma membrane[24, 25].

The strong expression of Kv3.4 in the stereocilia, the apical plasma membrane and the infracuticular zone of the mammalian auditory hair cells will provide the initial framework for the better understanding of the functional contribution of voltage-gated potassium channels to these cochlear sensory cells.

The results obtained in the present study suggest that the voltage-gated potassium channel Kv3.4 subunit in the cochlea may be involved in the potassium ion recycling mechanism in the inner ear. Further investigations are needed in order to elucidate the precise physiological role of the Kv3.4 in the cochlear function.

CONCLUSION

Immunohistochemical localization of a voltage-gated potassium channel Kv3.4 subunit was studied in the guinea pig cochlea. Intense Kv3.4-like immunoreactivity was observed in the type I and suprastrial fibrocytes of the spiral ligament and the basal cells of the stria vascularis. Immunostaining was also found in the type II, type III and type IV fibrocytes in the spiral ligament, supralimbal mesenchymal dark cells and fibrocytes in the spiral limbus. In the organ of Corti, Kv3.4-like immunostaining was found in the outer

hair cells, inner hair cells and the neighboring supporting cells. The root cells and interdental cells were also positively immunostained. Reaction product was also found in the endothelial cells of the blood vessels in the modiolus of the cochlea.

The results obtained in the present study suggest that the voltage-gated potassium channel, containing Kv3.4 subunit, may participate in the recycling system of potassium ions in the inner ear.

ACKNOWLEDGEMENTS

REFERENCES

1. Salt AN, Konishi T. The cochlear fluids: perilymph and endolymph. In: Altschuler RA, Hoffman DW and Bobbin RP (eds). *Neurobiology of Hearing: The Cochlea.* New York: Raven Press; pp. 109-122, 1986
2. Kikuchi T, Kimura RS, Paul DL et al. *Anat Embryol* 191: 101-118, 1995
3. Spicer SS, Schulte BA. *Hear Res* 100: 80-100, 1996
4. Kikuchi T, Kimura RS, Paul DL et al. *Brain Res Rev* 32: 163-166, 2000
5. Kikuchi T, Adams JC, Miyabe Y et al. *Med Electron Microsc* 33: 51-56, 2000
6. Kelsell DP, Dunlop J, Stevens HP et al. *Nature* 387: 80-83, 1997
7. Estivill X, Fortina P, Surrey S et al. *Lancet* 351: 384-398, 1998
8. Wangemann P. *Hear Res* 90: 149-157, 1995
9. So E, Kikuchi T, Ishimaru K et al. *NeuroReport* 12: 2761-2765, 2001
10. Neyroud N, Tesson F, Denjoy I e al. *Nature Genet* 15: 186-189, 1997
11. Sakagami M, Fukazawa K, Matsunaga T et al. *Hear Res* 56: 168-172, 1991
12. Kubisch C, Schroeder BC, Freidrich T et al. *Cell* 96: 437-446, 1999
13. Ando M and Takeuchi S. *Cell Tissue Res* 298: 179-183, 1999
14. Lecain E, Sauvaget E, Crisanti P et al. *Hear Res* 133: 133-138, 1999
15. Liang F, Hazen-martin D, Schulte BA et al. Abstracts of the 24th ARO Midwinter Research Meeting: 236, 2001
16. Vega-Saenz de Miera E, Weiser M, Kentros C et al. Shaw-related K$^+$ channels in mammals. In: Preacchia C (ed). *Handbook of membrane channels.* San Diego: Academic Press: pp 41-78, 1994
17. Conley EC. Vertebrate K$^+$ channels related to *Drosophila Shaw* (Kva subunits encoded by gene subfamily Kv3). In: Conley EC, Brammar WJ (eds). *The ion channel factsbook IV: Voltage-gated channels.* San Diego: Academic Press: pp. 559-616, 1999
18. Rettig J, Wunder F, Stocker M et al. *EMBO J* 11: 2473-2486, 1992
19. Xu C, Tang G, Lu Y et al. *Life Sci* 66: 2023-2033, 2000
20. Barrio LC, Suchyna T, Bargiello T et al. *Proc Natl Acad Sci USA* 88: 8410-8414, 1991

21. Verselis VK, Bargiello TA, Rubin JB et al. Comparison of voltage-dependent properties of gap junctions in hepatocytes and in *Xenopus* oocyte expression Cx32 and Cx26. In: Hall JE, Zampighi GA, Davis RM (eds). *Gap Junctions*. New York: Elsevier: pp. 105-112, 1993

22. Zhao HB, Santos-Sacchi J. Voltage gating of gap junctions in cochlear supporting cell: evidence for nonhomotypic channels. J Membr. *Biol* 175: 17-24, 2000

23. Rennie KJ, Ashmore JF. Ionic currents in isolated vestibular hair cells from the guinea-pig crista ampullaris. *Hear Res* 51: 279-292, 1991

24. Spicer SS, Thomopoulos GN, Schulte BA. Cytologic evidence for mechanisms of k⁺ transport and genesis of Hensen bodies and subsurface cisternae in outer hair cells. *Anat Rec* 251: 97-113, 1998

25. Spicer SS, Thomopoulos GN, Schulte BA. Novel membranous structures in apical and basal compartments of inner hair cells. *J Comp Neurol* 409: 424-437, 1999