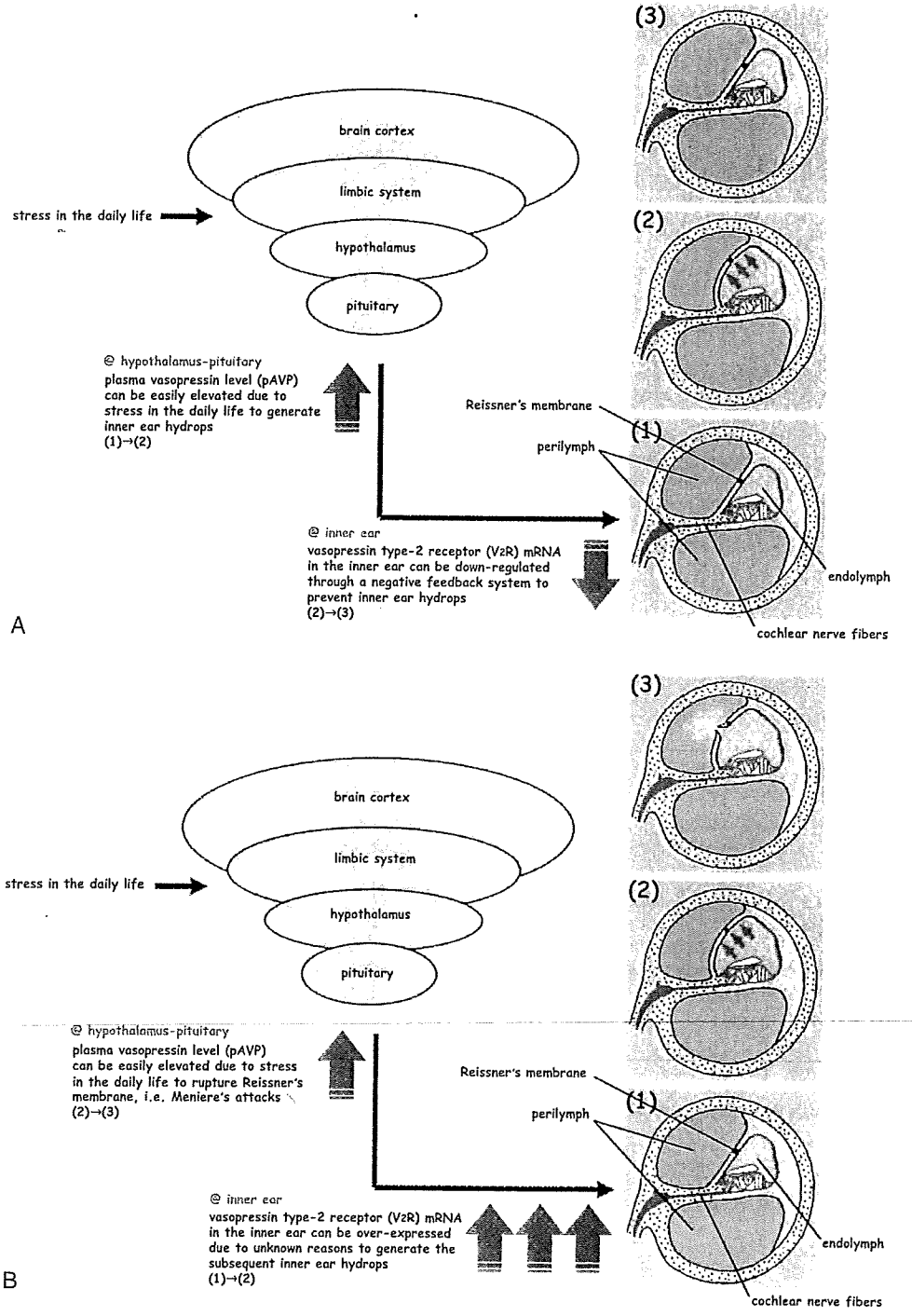

FIG. 3. Schematic representation of a hypothetical mechanism for endolymphatic hydrops generation and subsequent attacks of DEH and MD via pAVP and V2R. *A*, Healthy subjects: civilized people are frequently exposed to stress in their daily life, and pAVP can easily become elevated at any time (1)→(2). Therefore, a negative feedback system between pAVP and V2R in the inner ear may function for inner ear fluid homeostasis against stress-induced increases in pAVP (2)→(3). *B*, Patients with MD: autoimmune responses and/or virus infections in early childhood can lead V2R overexpression and hypersensitivity of cyclic AMP–linked signaling in the inner ear (1). After years, endolymphatic hydrops can gradually be generated (2). In the V2R-overexpressing and cyclic AMP–hypersensitized inner ear, the Reissner membrane can rupture after even a small elevation of pAVP due to stress, thereby resulting in attacks of MD (3). Upward arrows indicate upregulation; downward arrows, downregulation; number of arrows, strength of regulation.

Polymerase chain reaction products were electrophoresed on 3% Seakem GTG agarose gel (FMC Bioproducts, Rockland, ME, USA) and purified using QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Sequencing was accomplished by means of ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with ABI 310 DNA sequencer (Applied Biosystems).

Real-Time Quantitative PCR

Polymerase chain reactions were performed in the presence of the oligonucleotide primers for V2R (NM: 000054; forward: actgtgaggatgacgctagtgattg; reverse: ggacacgctgctgctgaaag) (Takara, Kyoto, Japan) and β_2-microglobulin (*B2M*) (NM: 004048; forward: cgggcatt cctgaagctga; reverse: ggatggatgaaacccagacacatag) (Takara) and quantified by SYBR Green PCR reagents (Applied Biosystems). *B2M*, an endogenous housekeeping gene, was used as an internal control for this method. Each sample determination was performed in triplicate.

The PCR mixture included 5 μl of 10× SYBR PCR buffer, 6 μl of 25 mmol/L $MgCl_2$, 4 μl of each deoxynucleotide triphosphate (blended with 2.5 mmol/L of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate and 5 mmol/L of 2′-deoxyuridine 5′-triphosphate), 2.5 μl of each gene-specific primer (5 μmol/L), 0.5 μl of AmpErase UNG (0.5 U), 0.25 μl of AmpliTaq Gold (1.25 U), and 5 μl (250 ng) of complementary DNA in a final volume of 50 μl. The conditions for the real-time PCR were as follows: 50°C for 2 minutes, 95°C for 12 minutes, and 35 cycles at 95°C for 15 seconds and 60°C for 1 minute in ABI PRISM 7700 Sequence Detection System (Applied Biosystems). 7700 Sequence Detection software was used for instrument control, automated data collection, and data analysis.

Data Analysis

The number of PCR cycles was recorded until the fluorescence intensity exceeded the predetermined threshold. The quantification of the initial amounts of template molecules relied on this number of PCR cycles, which is termed the cycle threshold (CT). The dCT represents the CT of the target gene normalized to the human endogenous *B2M* ($dCT = CT_{target} - CT_{B2M}$). Relative quantification of the mRNA expression levels of target genes (fold range) was calculated using the 2^{-ddCT} method, where $ddCT = (CT_{target} - CT_{B2M})_A - (CT_{target} - CT_{B2M})_B$ (12). For example, changes in the gene expression of V2R in endolymphatic sac in DEH compared with AN were quantified as the fold range: 2^{-ddCT} ($ddCT = [CT_{V2R} - CT_{B2M}]_{DEH} - [CT_{V2R} - CT_{B2M}]_{AN}$).

Western Blotting

Samples from the endolymphatic sac were homogenized on ice with a polytron homogenizer (PCU-11; Kinematica, Bohemia, NY, USA) in 20 mmol/L HEPES (pH 7.2), 25 mmol/L NaCl, 2 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 25 mmol/L β-glycerophosphate, 0.2 mmol/L dithiothreitol, 1 mmol/L phenylmethanesulfonyl fluoride, 60 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1% Triton X-100). After incubating at 4°C for 30 minutes, homogenates were sonicated (Sonifier 250; Branson Ultrasonics, Danbury, CT, USA) on ice for 1 minute and centrifuged at 10,000 × *g* at 4°C for 30 minutes. The supernatant was collected. Protein concentrations of these supernatants were measured with a protein assay kit (Pierce, Rockford, IL, USA). Gel samples were prepared by adding the sample buffer containing final concentrations of 50 mmol/L Tris (pH 6.7), 2% sodium dodecyl sulfate, and 2% mercaptoethanol. Twenty micrograms of protein extracts was boiled for 10 minutes, cooled to room temperature, and loaded on 10% sodium dodecyl sulfate–polyacrylamide gels. Equal amounts of protein in each sample were further checked by immunoblotting with β-actin monoclonal antibody (diluted to 1:500; Oncogene Research Products, Cambridge, MA, USA).

Proteins were transferred to Hybond–polyvinylidene difluoride membranes (Amersham, Piscataway, NJ, USA) by using standard electroblotting procedures. Membranes were incubated sequentially in the following solutions at 4°C: 2% nonfat dry milk, 1% bovine serum albumin (BSA) and normal goat serum (NGS) in 0.3% Triton X-100 in PBS for 3 hours; antisera against V2R (diluted to 1:500; sc-18100-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 24 hours; 0.1 mol/L PBS for 30 minutes; horseradish peroxidase–conjugated secondary antibody (Dako, Carpinteria, CA, USA) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 3 hours; 0.1 mol/L PBS for 30 minutes. Protein bands were visualized using an enhanced chemiluminescence detection kit and Hyperfilm MP (Amersham) and analyzed using Scion Image software (Scion Corp., Frederick, MD, USA).

STATISTICAL ANALYSIS

Statistical differences of patients' backgrounds (sex and age) among DEH, MD, and controls were examined by Kruskal-Wallis test among all 3 and then Mann-Whitney *U* test between each 2 (independent variables). Statistical differences of the data among DEH, MD, and controls in Figures 1 and 2 were determined by Kruskal-Wallis test among all 3 and then unpaired *t* test between each 2 (independent variables). Correlations between 2 parameters in Table 1 were analyzed by Pearson test. A multivariate analysis (a multiple regression analysis) was conducted to determine the relative predictive value of pAVP, V2R mRNA levels, and duration of DEH or MD for vertigo frequency. $p < 0.05$ were considered to indicate statistical significance. All the statistical analyses in the present study were performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

The raw data for 6 patients with ipsilateral DEH and 9 patients with unilateral MD, including their pAVP level,

V2R mRNA expression level in the endolymphatic sac, vertigo frequency (number of attacks per month), hearing level (four-tone average of 0.25, 0.5, 1, and 2 kHz) and duration of disease before surgery, are shown in Table 1.

The pAVP level was 1.5 times higher in patients with DEH (n = 20; 3.10 ± 0.58 pg/ml) than in control OM patients (n = 30; 2.11 ± 0.38 pg/ml), although this difference was not statistically significant (unpaired *t* test, $p = 0.140$; Fig. 1). There were no significant differences between the pAVP levels in patients with DEH and MD (n = 87; 3.47 ± 0.35 pg/ml; unpaired *t* test, $p = 0.636$).

The V2R mRNA expression level in the endolymphatic sac was 35.8 times higher in DEH patients (n = 6; 22.21 ± 0.58-fold) than in control AN patients (n = 6; 0.62 ± 0.10-fold), and this difference was statistically significant (unpaired *t* test, $p = 0.002$; Fig. 2A). These results were confirmed at the protein expression level by Western blotting (Fig. 2B). There were no significant differences between the V2R mRNA expression levels in patients with DEH and MD (n = 9; 27.86 ± 8.19-fold; unpaired *t* test, $p = 0.616$).

In both groups of patients with DEH (Pearson test, $r = -0.92$, $p = 0.009$) and MD (Pearson test, $r = -0.68$, $p = 0.043$), there were significantly negative correlations between pAVP and V2R mRNA expression in the endolymphatic sac (Table 1). A multivariate analysis (a multiple regression analysis) showed no significant predictive value of pAVP (DEH, $p = 0.87$; MD, $p = 0.78$), V2R mRNA levels (DEH, $p = 0.97$; MD, $p = 0.81$), or duration of DEH or MD (DEH, $p = 0.69$; MD, $p = 0.26$) for vertigo frequency. However, both pAVP (>2.5 pg/ml) and V2R mRNA expression in the endolymphatic sac (>10.00-fold) were relatively higher in Patients 4 and 5 with DEH and in Patients 6 to 8 with MD who experienced relatively more frequent vertigo attacks (≥4.0 attacks/mo).

DISCUSSION

For more than 10 years, it has been pointed out that the pAVP levels in patients with endolymphatic hydrops, including MD and DEH, during remission (13) as well as attacks (11), were significantly higher than those in patients with vertigo because of nonendolymphatic hydrops such as benign paroxysmal positional vertigo and vestibular neuronitis. It was also revealed that systemic injection of vasopressin induced bilateral endolymphatic hydrops and hearing deterioration in guinea pigs (14). These findings led us to the hypothesis that a high level of pAVP is one of the causes of inner ear endolymphatic hydrops in patients with MD and DEH. In contrast, however, it was reported that the pAVP levels in patients with unilateral MD did not differ significantly from those in healthy volunteers (15). Furthermore, the hypothesis of a high level of pAVP, which should have equal effects on both ears, contradicts the fact that 70% to 80% of patients with MD are unilateral (16). In fact, there were no significant differences between the pAVP levels in patients with unilateral and bilateral MD in our recent study (17). Therefore, the above hypothesis is insufficient for explaining the pathogenesis of endolymphatic hydrops, which has remained unsolved since endolymphatic hydrops was demonstrated in patients with MD in 1938 (3,4) and in patients with DEH in 1976 (2).

Regarding vasopressin receptors, V2R molecules have been detected in rat (18,19) and human (18,20) inner ear end organs. V2R was clearly distributed together with a V2R-linked water channel molecule, aquaporin 2, in the luminal epithelium of the human endolymphatic sac (20). Interestingly, the physiological interactions between pAVP and V2R in the endolymphatic sac attenuated the membranous turnover via cyclic AMP–dependent signaling in a contrasting manner with the kidney in rats (18), and then these interactions also translocated aquaporin 2 from the luminal side to the basolateral side in a contrasting manner with the kidney in human (our unpublished observations). These previous findings indicate that V2R and cyclic AMP–linked signaling could suppress the endolymphatic fluid absorption in the endolymphatic sac.

In the present study, we first detected 35.8-fold higher V2R mRNA expression in the endolymphatic sac in patients with ipsilateral DEH compared with controls, whereas the pAVP level was only elevated by 1.5 times. There were no significant differences in the pAVP or V2R expression in the endolymphatic sac between ipsilateral DEH and unilateral MD. These findings in the present study suggest that V2R overexpression in the endolymphatic sac in ipsilateral DEH as well as unilateral MD could attenuate the membrane turnover and cause the endolymphatic fluid overflow into the endolymphatic space after even a small increase in pAVP. In other words, patients with unilateral V2R overexpression in the endolymphatic sac could develop unilateral endolymphatic hydrops, resulting in attacks of vertigo in the overexpressing ear after even a small elevation in pAVP due to stress in their daily life.

Second, we detected a significantly negative correlation between pAVP and V2R expression in the endolymphatic sac in DEH as well as MD, consistent with the previous study on intact rats (19). These findings suggest that a negative feedback system between pAVP and its receptor in the endolymphatic sac could function for inner ear fluid homeostasis against stress-induced increases in pAVP (Fig. 3A). A negative feedback system between the hypothalamus-pituitary and inner ear has not been clarified yet. However, the direct neuroanatomic interactions between the hypothalamus-pituitary and inner ear were demonstrated electrophysiologically (21,22) and morphologically (23). Inner ear volume and/or pressure changes modified the pAVP secretion (24–26), which indicates that the negative feedback system is really working. Anyway, this negative correlation may explain why the previous studies of pAVP in MD and DEH produced a large variety of results and have been quite controversial (10, 11,13,15).

Finally, we would like to speculate about the possible causes for attacks associated with inner ear abnormality in

MD and DEH. It has been reported that MD is usually triggered by immune, infectious, traumatic, or other insults to the inner ear in association with a small misplaced malfunctioning endolymphatic sac (27–29). Among these insults, immune-mediated responses in the inner ear end organs, such as the endolymphatic sac, stria vascularis, and spiral ligament, are thought to be the main bases for the fluid homeostatic disorder in MD (30,31). Certain virus infections, such as varicella-zoster, Epstein-Barr, and adenovirus infections, of the endolymphatic sac in early childhood represent other bases for the dysfunction of endolymph absorption (32,33). Taken together with the present data, it is suggested that autoimmune responses and/or virus infections could modify the V2R regulatory system in the endolymphatic sac, resulting in V2R overexpression there (Fig. 3B-1). Years after such insults to the inner ear, hardworking people tend to become frequently exposed to stress in their daily life (1). In the V2R-overexpressing inner ear, endolymphatic hydrops could gradually be generated (Fig. 3B-2), and the Reissner membrane could rupture (2) after even a small elevation in pAVP due to stress, thereby resulting in attacks of MD and DEH (Fig. 3B-3).

CONCLUSION

Civilized people are frequently exposed to stress in their daily life, and pAVP can easily become elevated at any time. Therefore, a negative feedback system between pAVP and its receptor, V2R, in the endolymphatic sac may function for inner ear fluid homeostasis against stress-induced increases in pAVP. For the pathogenesis of endolymphatic hydrops resulting in vertigo attacks in MD and DEH, pAVP may represent a matter of consequence, but V2R overexpression in the endolymphatic sac could be much more essential as a basis for these diseases.

The results of the present study encourage us to continue our investigations and ascertain ideal treatments for the inner ear in patients with DEH as well as MD, ranging from psychotherapy for leading a stressless life to gene therapy for the stress hormone receptor V2R regulation in the inner ear.

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ORIGINAL ARTICLE

Tinnitus as a prognostic factor of sudden deafness

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Abstract

Conclusions. The 'tinnitus-rare' group had a poorer prognosis for hearing than the 'tinnitus-often' group in all sudden sensorineural hearing loss (SSNHL), although the 'shorter duration' group had better prognosis than the 'longer duration' when restricted to SSNHL accompanied by tinnitus. This indicates that tinnitus itself may not be a sign for poor hearing prognosis but might be an essential sound for the initiation of repair of a damaged auditory system. *Objectives.* We examined the hearing improvement rate (HIR) and tinnitus at the onset of SSNHL to elucidate the prognostic value of tinnitus accompanying SSNHL. *Patients and methods.* Fifty patients with SSNHL were treated with systemic administration of steroids. Hearing recovery was determined by comparing the hearing levels before and after treatment. Tinnitus was subjectively evaluated by the tinnitus scoring questionnaire. The score for the five-step evaluation of the subjective tinnitus feelings 'loudness', 'duration' and 'annoyance' was obtained at the onset. *Results.* In terms of 'duration', when we divided all the cases into 'tinnitus-rare' group and 'tinnitus-often' group, HIR in the 'tinnitus-rare' group was significantly lower than that in 'tinnitus-often' group. When restricted to the 'tinnitus-often' group, HIR for 'shorter duration' was significantly higher than that for 'longer duration'.

Keywords: *Sudden deafness, vertigo, tinnitus, hearing improvement rate, prognostic factor*

Introduction

Sudden sensorineural hearing loss (SSNHL) is defined as a sensorineural hearing loss of 30 dB or worse in three consecutive speech frequencies that has occurred with sudden onset [1,2]. The incidence of SSNHL is estimated to range from 5 to 20 per 100 000 population [2]. Various kinds of causes of SSNHL have been suggested as follows: viral infection of labyrinth or cochlear nerve, vascular insult, intralabyrinthine membrane rupture and perilymphatic fistula [1].

To date, some prognostic factors for SSNHL have often been reported. Vertigo, particularly severe vertigo, has been considered as a negative prognostic factor [1–3]. Delayed start of treatments after the onset has also been considered as a negative prognostic factor [2,4]. Byl Jr also reported that age over 60 years or below 15 years has been considered a negative prognostic factor [2]. Additionally, the severity of the initial hearing level has been considered a negative prognostic factor [1,2,5,6]. As regards tinnitus, although several papers on this topic have already been published, the prognostic value has been controversial. Wilson et al. and Moskowitz et al. reported that tinnitus was a negative prognostic factor for hearing after SSNHL [7,8]. Cadoni et al. also described that hearing recovery was poorer in SSNHL patients with both tinnitus and vertigo than those with only vertigo [5]. While Byl Jr reported that tinnitus had little prognostic value [2], Danino et al. reported that tinnitus was a favourable prognostic manifestation in an analysis of symptoms and recovery rates in 60 patients with SSNHL [9]. Ben-David et al. found tinnitus to be strongly associated with hearing improvement in 67 patients with SSNHL and indicated that tinnitus was a positive prognostic factor for hearing after SSNHL [10].

In the present study, to elucidate the relationship between hearing prognosis and tinnitus at the onset

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of SSNHL, we examined changes in hearing and tinnitus of patients with SSNHL using pure-tone audiometry (PTA) and the tinnitus scoring questionnaire [11].

Patients and methods

Charts of 50 consecutive patients with the diagnosis of SSNHL accompanying vertigo (25 females and 25 males, mean 48.6 years, range 11–80 years) attending the Dizziness & Vertigo Section of the Department of Otolaryngology in three hospitals (Osaka Rosai Hospital, Osaka University Hospital and Tondabayashi Hospital) from 1998 to 2007 were reviewed in this retrospective study. Patients with Meniere's disease were carefully excluded.

All the patients underwent PTA, electronystagmography (ENG) and MRI for the purpose of excluding possible retrocochlear lesions, including demyelinating diseases, at the first visit. All the treatments started within 7 days after the onset (2.8 ± 1.7 days), including bed rest and intravenous applications of hydrocortisone sodium succinate (from 500 mg/day with dose reductions of 200 mg every 3 days to zero) and lasted for 1–2 weeks at most.

Hearing recovery was determined by comparing the audiometric results at the first visit (2.8 ± 1.7 days: pretreatment) and the last visit approximately 6 months later, when hearing function was assumed to be fixed completely (6.6 ± 1.3 months: post-treatment). The hearing improvement rate (HIR) was used as a credible parameter for hearing recovery after SSNHL [12]. Hearing gain was an absolute value of changes in averaged hearing levels of 250, 500, 1000, 2000 and 4000 Hz from pretreatment to post-treatment. HIR was defined as a result of hearing gain divided by differences between averaged initial hearing levels in the affected and unaffected ear, multiplied by 100.

Tinnitus was subjectively evaluated by the tinnitus scoring questionnaire according to the Tinnitus Research Group of Japan Audiological Society (TRGJ) in 1993 (Table I) [11]. The score of five-step evaluation from 1 to 5 in the three items of subjective tinnitus feelings – 'loudness', 'duration' and 'annoyance' – was obtained from patients at the onset of SSNHL.

For neuro-otologists, it is very important to tell patients with SSNHL their hearing prognosis at the first visit. Therefore, we examined the relationship between tinnitus score at the onset and HIR.

Statistical analysis was performed based on the Mann-Whitney test and Spearman correlation test. All reported p values were two-sided and those under 0.05 were considered to be significant.

Results

The number of patients for each item of the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance', is summarized in Figure 1.

(i) Tinnitus score pretreatment and HIR

None of three items in the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance', was significantly related to HIR according to the Spearman correlation test. As no one selected score 3 for 'duration' (Figure 1), we divided the pretreatment tinnitus scores for 'duration' into two groups. We defined scores 1 and 2 as 'tinnitus-rare' and scores 4 and 5 as 'tinnitus-often'. HIR for the 'tinnitus-rare' group ($n=8$; $16.3 \pm 34.0\%$, range -5.0 to 100.0%) was significantly lower than that for the 'tinnitus-often' group ($n=42$: $53.2 \pm 35.4\%$, range -19.0 to 119.0%) (Mann-Whitney: $U=72.0$, $p=0.011 < 0.05$) (Figure 2A). When restricted to the 'tinnitus-often' group ($n=42$), we compared the HIR for score 4, 'shorter duration' and score 5, 'longer duration'. HIR with 'shorter duration' of tinnitus ($n=9$: $99.5 \pm 9.5\%$, range 88.0–119.0%) was significantly higher than that for 'longer duration' of tinnitus ($n=33$: $40.6 \pm 29.3\%$, range -19.0 to 100.0%) (Mann-Whitney: $U=5.0$, $p=1.17\text{E-}05 < 0.001$) (Figure 2B).

(ii) Tinnitus score pretreatment and hearing level pretreatment

None of three items in the pretreatment tinnitus score was significantly related to pretreatment hearing level

Table I. Tinnitus scoring questionnaire: Tinnitus Research Group of Japan Audiological Society, 1993.

	Score				
Parameter	1	2	3	4	5
Loudness	Very quiet	Quiet	Medium	Loud	Very loud
Annoyance	Not at all	Slightly	Frequent	Always	Very much
Duration	Rare	Less often	Often	Very often	Constant

The nature and severity of tinnitus were evaluated in 5 steps from 1 to 5 in the three different categories of subjective tinnitus feelings – 'loudness', 'duration' and 'annoyance'.

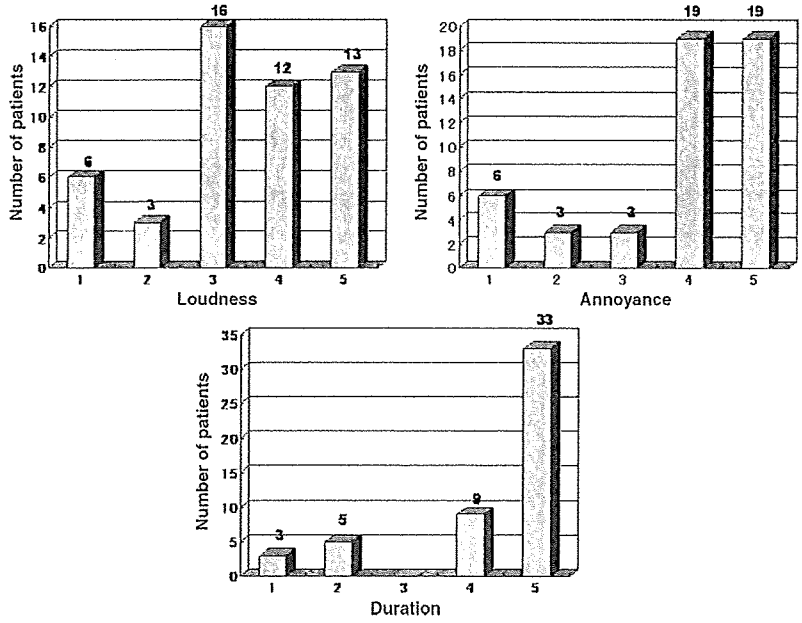

Figure 1. Results of pretreatment tinnitus score. The population in each item of the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance' was summarized.

according to the Spearman correlation test. When we divided pretreatment tinnitus scores of 'duration' into 'tinnitus-rare' and 'tinnitus-often' groups as above, the pretreatment hearing level in the 'tinnitus-rare' group ($n=8$: 86.0 ± 23.5 dB, range 49.0–115.0 dB) was not different from that in the 'tinnitus-often' group ($n=42$: 74.5 ± 24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: $U=120.0$, $p=0.208$) (Figure 3A). When restricted to the 'tinnitus-often' group ($n=42$), the pretreatment hearing level for score 4, 'shorter duration' of tinnitus ($n=9$: 55.0 ± 11.1 dB, range 35.0–69.0 dB) was significantly better than that for score 5, 'longer duration' of tinnitus ($n=33$: 79.8 ± 24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: $U=63.0$, $p=0.009<0.01$) (Figure 3B).

(iii) Tinnitus score pretreatment and age of patients

When we divided pretreatment tinnitus scores of 'duration' into 'rare' and 'often' groups as in (i), the age of patients in the 'tinnitus-rare' group ($n=8$: 46.4 ± 22.3 years, range 11–70 years) was not different from that in the 'tinnitus-often' group ($n=42$: 49.1 ± 16.4 years, range 15–80 years) (Mann-Whitney: $U=164.5$, $p=0.937$). When restricted to the 'tinnitus-often' group ($n=42$), the age of patients with score 4, 'shorter duration' of tinnitus ($n=9$: 37.6 ± 8.8 years, range 25–52 years) was significantly younger than that with score 5, 'longer duration' of tinnitus ($n=33$: 52.2 ± 16.7 years, range 15–80 years) (Mann-Whitney: $U=59.5$, $p=0.007<0.01$).

Discussion

According to previous studies, tinnitus was reported to accompany SSNHL in 74–87% of patients [2,6,13]. In the present study, 88% of SSNHL patients with vertigo complained of tinnitus. In Japan, tinnitus is usually evaluated by the tinnitus scoring questionnaire according to the TRGJ (1993) (Table I) [11]. This questionnaire is easy to handle but does not really provide an objective evaluation. However, among the three items of subjective tinnitus feelings, only 'duration' was significantly correlated with HIR (cf. (i) in Results). This finding suggests that 'duration' could be the most reliable item for tinnitus evaluation of patients with SSNHL. As tinnitus is a quite subjective symptom, the most reliable way of evaluation in the present study could change the status of tinnitus from a non-evaluable complaint of patients to an important prognostic factor for SSNHL.

As summarized in the Introduction, the prognostic value of tinnitus in SSNHL has been controversial until now [2,5,7–10]. In the present study, 'shorter duration' of tinnitus was a positive prognostic factor for hearing after SSNHL, as reported by Cadoni et al. [5], Wilson et al. [7] and Moskowitz et al. [8]. Our data also suggested that 'tinnitus-rare' was a negative prognostic sign for results of treatments of SSNHL, as described by Danino et al. [9] and Ben-David et al. [10]. Taking all these facts together, we assume that tinnitus may lose its prognostic value absolutely, as in the paper by Byl Jr [2], when 'shorter duration' and 'tinnitus-rare' are completely mixed up.

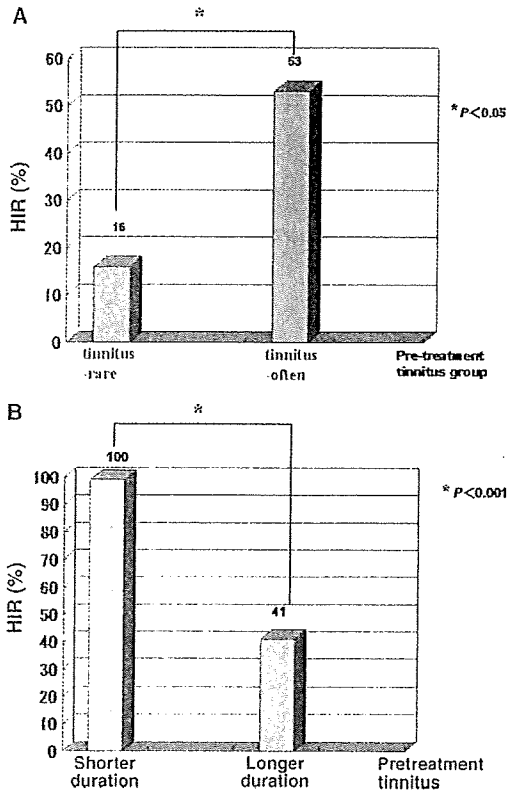

Figure 2. (A) Tinnitus score pretreatment and hearing improvement rate (HIR). The HIR for the 'tinnitus-rare' group (score 1–2) ($n=8$: 16.3±34.0%, range −5.0 to 100.0%) was significantly lower than that for the 'tinnitus-often' group (score 4–5) ($n=42$: 53.2±35.4%, range −19.0 to 119.0%) (Mann-Whitney: $U=72.0$, $p=0.011<0.05$). (B) When restricted to the 'tinnitus-often' group ($n=42$), the HIR for 'shorter duration' of tinnitus (score 4) ($n=9$: 99.5±9.5%, range 88.0–119.0%) was significantly higher than that for 'longer duration' of tinnitus (score 5) ($n=33$: 40.6±29.3%, range −19.0–100.0%) (Mann-Whitney: $U=5.0$, $p=1.17E\text{-}05<0.001$).

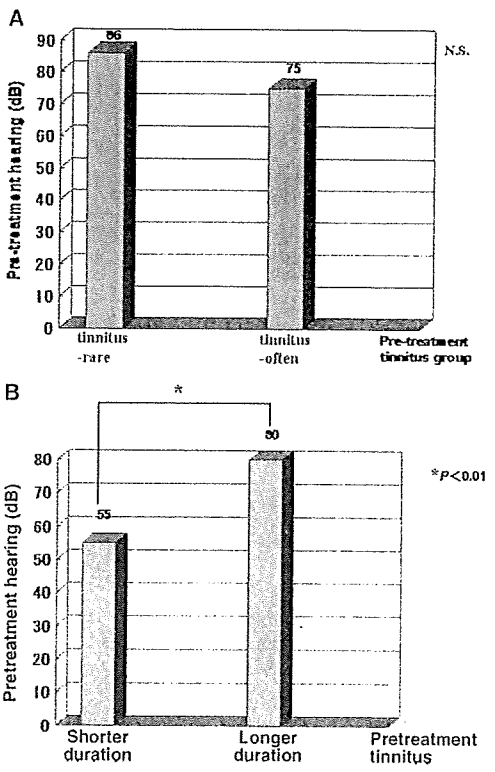

Figure 3. Tinnitus score and hearing level pretreatment. (A) The pretreatment hearing level for the 'tinnitus-rare' group (score 1–2) ($n=8$: 86.0±23.5 dB, range 49.0–115.0 dB) was not significantly different from that of the 'tinnitus-often' group (score 4–5) ($n=42$: 74.5±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: $U=120.0$, $p=0.208$). (B) When restricted to the 'tinnitus-often' group ($n=42$), the pretreatment hearing level for 'shorter duration' of tinnitus (score 4) ($n=9$: 55.0±11.1 dB, range 35.0–69.0 dB) was significantly better than that for 'longer duration' of tinnitus (score 5) ($n=33$: 79.8±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: $U=63.0$, $p=0.009<0.01$).

When tinnitus was present at the onset of SSNHL, the 'shorter duration' group had a better HIR than the 'longer duration' group (cf. (i) in Results). This may come from the fact that tinnitus duration was significantly shorter in better hearing (cf. (ii) in Results) and/or younger (cf. (iii) in Results) patients at the onset. These findings suggest that the duration of tinnitus and the amount of damage in the inner ear could have a positive relationship in patients with SSNHL accompanied by tinnitus, resulting in a negative relationship with HIR.

On the other hand, the 'tinnitus-rare' group had a poorer HIR than the 'tinnitus-often' group in all the patients with SSNHL (cf: (i) in Results). This may indicate that tinnitus itself may not be a sign for poor hearing prognosis but might be an essential sound for cell survival. Actually, the tinnitus research group of Kitahara and Balaban [14,15] demonstrated that high doses of salicylate could up-regulate a neurotrophic factor, brain-derived neurotrophic factor (BDNF), in the inner ear for cell survival and lead subsequent transcription of a nociceptive cation ion channel receptor, transient receptor potential cation channel superfamily V type 1 (TRPV1) in the inner ear for tinnitus generation. These findings suggest the hypothesis that tinnitus might be a switch-on signal for inner ear cell survival. According to this hypothesis, it could be speculated that causes and/or sites of lesion in SSNHL without tinnitus are absolutely different from those in SSNHL with tinnitus, which is one of the reasons why the prognostic value of tinnitus in SSNHL has been controversial until now [2,5,7–10].

Conclusion

In conclusion, tinnitus at the onset of SSNHL is important as a prognostic factor for hearing. 'Tinnitus-often' is a positive prognostic sign for

hearing recovery, but 'longer duration' predicts poor results in hearing improvement. Further detailed investigation of tinnitus at the time of SSNHL may elucidate mechanisms of tinnitus generation and may lead to development of effective treatments for tinnitus.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Analysis of gene expression profiles along the tonotopic map of mouse cochlea by cDNA microarrays

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Abstract

Conclusion: This study demonstrated differential gene expression profiles along the axis of the mouse cochlea. It also suggests the mechanism that establishes the tonotopic organization. *Objectives:* The molecular basis of the tonotopic mapping of the mammalian cochlea remains unclear. We therefore examined the genes that were abundantly expressed in either the apex or the base of mouse cochlea. *Materials and methods:* Apical and basal portions of cochlea, which contained the organ of Corti and spiral ganglion neurons, were independently dissected from 10 adult C57BL/6 mice, and their total RNAs were respectively isolated. The gene expression profiles of each of these two pools were examined and compared to each other by the complementary DNA microarray technique. *Results:* Of 20 289 probes tested, 64 genes were found to be expressed in the apical portion more than fivefold more abundantly than in the basal portion, and 77 genes were vice versa. Of interest, the genes of several functional proteins such as β2- and γ2-subunits of $GABA_A$ receptors dominated the basal part, indicating that these molecules may be involved in high-frequency tuning of the hair cells and/or ganglion neurons.

Keywords: *Cochlea, tonotopic mapping, cDNA microarray, gene profile, mouse*

Introduction

The cochlea of the inner ear is the peripheral auditory organ that converts vibrations of sounds to electrical signals and transmits them to the central nervous system. These tasks are achieved mainly by hair cells, which are the primary receptors for hearing and the constituents of the organ of Corti [1–3]. The mechanical stimulation by sounds opens mechano-sensitive channels at the tip of the hair cells' stereocilia, which allows influx of K^+ from endolymph to the cells and excites them. Afterwards, a neurotransmitter glutamate is released from the hair cells and stimulates the dendrites of the spiral ganglion neurons [2]. The neurons further activate auditory nuclei of brain via their axons. Therefore, two components of the cochlea, i.e. the organ of Corti containing hair cells and the ganglion neurons, are essential for hearing.

One of the features of the cochlea is that this organ is tonotopically orientated: the hair cells at the apical portion are sensitive to low frequencies and those at the basal side vice versa. Previous studies suggested that this monotonic tuning map involved various elements in different species. In amphibians, reptiles, and birds, stimulation of hair cells caused the oscillation of their membrane potential. Its frequency, called 'electrical resonant frequency,' is unique to a hair cell at a respective portion along the axis of the cochlea. The oscillation serves as the electrical filter to maximize a hair cell's response to sounds of particular frequencies and is at least partially determined by differential expression of variants of Ca^{2+}-activated K^+ channels along the tonotopic map [4–7]. The length of hair bundles is longer in the hair cells of the apex, the mechanism that tunes the bundles sensitive to lower frequencies. Furthermore, synaptic vesicles are specialized to fuse a membrane of the hair cell most efficiently at its characteristic frequencies [8]. In mammals, the stiffness of the basilar membrane varies along the cochlear axis, which plays a pivotal role in amplifying a specific frequency of sound-induced vibration in

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each portion [9]. The adaptation time constant of mechano-electrical transduction current becomes faster from the apex to the base, which imposes a bandpass filter on transduction and improves the signal-to-noise ratio near threshold [10]. In spite of this evidence, the precise machinery determining tonotopic organization is still poorly understood.

To identify the functional molecules that underlie the tuning property of the mammalian cochlea, in this study we examined and compared gene expression profiles in the organ of Corti and spiral ganglion neurons between the apical and basal portions of the cochlea by microarray technology. The genes that were found to be differentially expressed along the tonotopic map were of the proteins involved in a variety of signal transductions such as ionic transports, neurotransmission, and protein modification.

Materials and methods

Tissue dissection and RNA extraction

All animals were handled in accordance with the guidelines of the Animal Care Committee of Osaka University Medical School. Ten C57BL/6 mice aged 6 weeks were euthanized by decapitation, and wetted with 70% ethanol to prevent contamination of samples with RNase-rich hairs. Cochleae were rapidly removed from the temporal bone and transferred to a 5 ml bath of RNA-later solution (Ambion, Austin, TX, USA). After removing the otic capsule, spiral ligament, and stria vascularis from the cochlea, the organ of Corti and modiolus were dissected and separated into apical and basal segments. All of these procedures were performed in RNA-later solution on a chilled plate to prevent RNA degradation. The pooled samples were homogenized with a micro-homogenizer (Microtec, Chiba, Japan) for 15 s, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of extracted total RNA was confirmed with bioanalyzer biosizing gel in which the 28S, 18S, and 5S ribosomal bands were clearly visible (data not shown) by using an Agilent Technologies 2100 Bioanalyzer-Bio Sizing (Agilent Technologies, Palo Alto, CA, USA).

RNA labeling and microarray hybridization

Experiments were conducted according to the manufacturer's instructions for CodeLink Expression Bioarrays (Amersham Bioscience, Piscataway, CA, USA). Total RNA was used to produce single-stranded cDNA in the presence of T7-24 Oligo(dT) promoter primer and reverse transcriptase (RT). After double-stranded cDNA synthesis with DNA polymerase I and RNase H, the cDNA was prepared as the template in an *in vitro* transcription reaction to produce the target cRNA. The *in vitro* transcription reaction was performed in the presence of biotinylated nucleotides to label the target cRNA. A bacterial mRNA was used as positive control for the cDNA synthesis and in vitro transcription reaction. Each step of the procedure was monitored using these control mRNAs.

The biotin-labeled cRNA was fragmented randomly by incubation in the presence of magnesium for 20 min at 94°C. Fragmented target cRNA (10 μg) was used for hybridization with a CodeLink Uniset Mouse 20K Bioarray chip (Amersham), which was spotted by 20 289 probes including 108 positive control genes and 300 negative control genes.

Microarray analysis

Hybridized microarray chips were washed and processed using a direct detection method of the biotin-labeled transcripts by Streptavidin-Cy5 conjugates. Slides were scanned by CodeLink scanning software (Motorola Life Sciences, Pasadena, CA, USA). Images for each slide were quantified using the CodeLink expression analysis software (Motorola Life Sciences). The signal intensity for each spot was calculated by summation of the pixel intensities in each spot, and subtracting the local background (based on the median pixel intensity of the area surrounding each spot). In each slide, signal intensities were normalized by dividing the intensity of each spot (after background subtraction) by the median signal intensity of all spotted probes.

Results

Total RNAs isolated from either the apical or basal portion of the cochlea were assayed by cDNA microarray technique. We tested 20 289 probes for each experiment. When the signal intensity of the probe in the apex was more than fivefold stronger or weaker than that of the base, we concluded that the difference was significant. We identified that 64 probes provided higher intensity for the pool of the apical part than for that of the basal part. Of these genes, the products of 39 genes (61%) had been named whereas the others were categorized to expressed sequence tag (EST) sequences (25 genes: 39%) (Figure 1a). On the other hand, we found that 77 genes were much more abundant in the base than in the apex: 60 genes (78%) had been named and 17 genes (22%) belonged to EST sequences (Figure 1b). We classified some of the genes, of which products had been physiologically characterized, into functional categories (Tables I and II). Accession

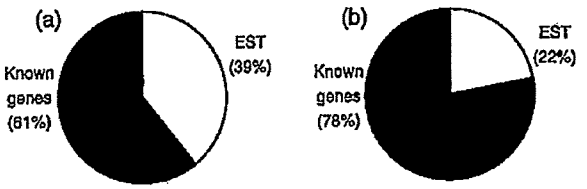

Figure 1. Classification of genes. (a) Sixty-four genes with higher expression in the apex. Of these genes, 39 genes (61%) had been named whereas the others were categorized to expressed sequence tag (EST) sequences (25 genes, 39%). (b) Seventy-seven genes with higher expression in the base. 60 genes (78%) had been named and 17 genes (22%) belonged to EST sequences.

numbers of the aforementioned EST sequences are shown in Table III.

Of the genes dominant in the base, five genes were of ion transporters and the products of seven genes were related to neurotransmission. In contrast, the group of the genes highly expressed in the apex contained only one cDNA of ion transporter and nothing associated with neurotransmission.

Discussion

Using the cDNA microarray technique we determined that 141 genes were differentially expressed between the apical and the basal portions of the cochlea. Although the expression pattern of mRNA does not always correlate with that of the protein, our finding may provide a mechanistic insight into the system underlying the tonotopic organization of the mammalian cochlea.

Among the highly expressed gene in the base, five genes were in the ion transporter group and seven genes were related with neurotransmitter, while among the highly expressed genes in the apex, only one gene was classified as an ion transporter and there were no genes associated with neurotransmitters. It is conceivable that the basal, high-frequency neurons

Table I. Genes showing higher expression in the apex by categories.

Function	Accession no.	Gene	Ratio (apex:base)
Structural cytoskeleton	NM_009406.1	Troponin I, cardiac (TNNI 3)	10.6
	NM_010867.1	Myomesin 1 (MYOM1)	5.3
	AJ002521.1	Myosin heavy chain 2A (MYH2)	5.3
	NM_010052.1	Delta-like 1 homolog (Drosophila) (DLK1)	7.1
Cell adhesion	NM_008402.1	Integrin alpha V (CD51) (ITGAV)	9.3
Membrane transport	NM_008429.1	Potassium inwardly-rectifying channel, subfamily J, member 9 (KCNJ9)*	14.9
	NM_021889.2	Synaptotagmin 9 (SYT9)	5.8
	NM_009205.1	Solute carrier family 3, member 1 (SLC3A1)	12.1
Transcription factor or nucleic acid synthesis and modification	NM_011623.1	Topoisomerase (DNA) II alpha (TOP2A)	5.5
	NM_009392.1	T-cell leukemia, homeobox 2 (TLX2)	6.1
	NM_010569.2	Inversin (INVS)	7.4
	NM_009238.1	Sry-box containing gene 4 (SOX4)	6.0
	NM_010635.1	Kruppel-like factor 1 (Erythroid) (KLF1)	5.4
	NM_053111.1	Eosinophil-associated ribonuclease 6 (EAR6)	5.0
	NM_007894.1	Eosinophil-associated ribonuclease 1 (EAR1)	5.7
Protein modification	NM_008079.1	Galactosylceramidase (GALC)	7.1
	NM_019775.2	Carboxypeptidase B2 (CPB2)	8.8
	NM_145217.1	Diras family, GTP-binding ras-like 1 (DIRAS1)	5.5
Signal transduction	NM_011611.1	Tumor necrosis factor receptor superfamily, member 5 (TNFRSF5)	5.3
Hormone	NM_008117.1	Growth hormone (GH)	7.1
	NM_009889.1	Glycoprotein hormones, alpha subunit (CGA)	20.0
	NM_011164.1	Prolactin (PRL)	12.6
Immune or inflammatory response	NM_018866.1	Chemokine (C-X Motif) ligand 13 (CXCL13)	6.0
	NM_008327.1	Interferon activated gene 202A (IFI202A)	5.6
Others	NM_010841.1	Metallothionein-like 5, testis-specific (TESMIN) (MTL5)	10.1
	NM_013868.2	Heat shock protein family, member 7 (CARDIOVASCULAR) (HSPB7)	6.7

*Genes related to ion exchange.

Table II. Genes showing higher expression in the base by categories.

Function	Accession no.	Gene	Ratio (apex:base)
Structural cytoskeleton	NM_028514.1	Riken cDNA 1700061J02 gene (ACTRT1-pending)	5.8
	NM_021541.1	Crystallin, beta A2 (CRYBA2)	5.7
	NM_008614.2	Myelin-associated oligodendrocytic basic protein (MOBP)	8.0
	NM_009789.1	Calbindin 3 (vitamin D-dependent calcium binding protein) (CALB3)	9.2
Cell adhesion	NM_011581.1	Thrombospondin 2 (THBS2)	7.5
	NM_011707.1	Vitronectin (VTN)	5.0
	W53823.1	MD14C10.R1 soares embryo NBME 13.5 14.5 cDNA clone (COL6A2)	5.3
	NM_022031.1	Brain-like protein 1 (BRAL1-pending)	8.1
Membrane transport	NM_133241.1	Megalencephalic leukoencephalopathy with subcortical cysts 1 homolog (HUMAN) (MLC1)*	5.8
	NM_013667.1	Solute carrier family 22 (organic cation transporter), member 2 (SLC22A2)*	5.2
	NM_031194.2	Solute carrier family 22 (organic anion transporter), member 8 (SLC22A8)*	9.3
	NM_054055.1	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (SLC13A3)*	6.0
	NM_148931.2	Solute carrier family 6 (neurotransmitter transporter, glycine), member 5 (SLC6A5)†	5.3
	NM_010018.1	D-Amino acid oxidase (DAO1)	10.8
	AK005250.1	Adult male cerebellum cDNA, riken clone 1500015I14 product, Solute carrier family 25 (SLC25A18)	5.5
	NM_026205.2	Riken cDNA 1700010O16 gene (1700010O16RIK)*	5.1
	NM_133661.1	Gamma-aminobutyric acid (GABA-A) transporter 2 (GABT2) (SLC6A12)†	5.9
	NM_144512.1	Gamma-aminobutyric acid (GABA-A) transporter 3 (GABT3) (SLC6A13)†	6.6
	NM_008070.2	Gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2 (GABRB2)*†	14.3
	NM_008073.1	Gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2 (GABRG2)*†	6.8
	NM_008747.1	Neurotensin receptor 2 (NTSR2)†	7.2
Transcription factor	NM_023729.2	Germ cell-specific ankyrin, sam and basic leucine zipper domain containing protein (GASZ-pending)	5.7
	NM_009573.1	Zinc finger protein of the cerebellum 1 (ZIC1)	8.7
	NM_010919.1	NK2 transcription factor related, locus 2 (Drosophila) (NKX2-2)	6.8
Protein modification	NM_008294.1	Hydroxysteroid dehydrogenase-4, delta-3-beta (HSD3B4)	5.4
	L20331.1	Adenosin-3 receptor homolog (ADORA3)	16.7
	NM_016672.1	Aromatic-L-amino-acid decarboxylase (DDC)	5.6
	NM_011219.1	Protein tyrosine phosphatase, receptor type, Z polypeptide 1 (PTPRZ1)	6.0
	NM_007607.1	Carbonic anhydrase 4 (CAR4)	10.3
	NM_018870.1	Phosphoglycerate mutase 2 (PGAM2)	16.3
	NM_011177.1	Protease, serine, 18 (PRSS18)	6.2
	NM_008223.1	Serine (or cysteine) proteinase inhibitor, clade D, member 1 (SERPIND1)	5.9
Signal transduction	BB451927.2	Riken, 12 days embryo spinal ganglion cDNA clone D130013H04 3 (SSTR4)	5.5
Hormone	NM_031161.1	Cholecystokinin (CCK)	5.5
	NM_011702.1	Vasoactive intestinal polypeptide (VIP)	6.6
Inflammatory response	NM_011260.1	Regenerating islet-derived 3 gamma (REG3G)	5.1
Others	NM_008013.2	Fibrinogen-like protein 2 (FGL2)	5.4
	NM_008579.3	Meiosis expressed gene 1 (MEIG1)	6.8
	NM_010923.1	Neuronatin, transcript variant 1 (NNAT)	6.5
	NM_134238.1	Vomeronasal 1 receptor, H13 (V1RH13)	6.3
	NM_009311.1	Tachykinin 1 (TAC1)†	20.8

*Genes related to ion exchange.
†Genes related to neurotransmitter.

Table III. List of all expressed sequence tags (ESTs).

Accession nos of ESTs that showed higher expression in apex					
AI850080.1	AK010233.1	AV246932.2	BB042553.2	BB044797.2	BB078328.1
BB078496.1	BB081209.1	BB085099.2	BB113917.1	BB160318.2	BB308379.2
BB334120.2	BB339785.2	BB444977.1	BB475313.1	BB539070.2	BB695600.1
BE456549.1	BE952696.1	BG806739.1	BM234411.1	BM248364.2	BM248364.2
NM_008386.2					
Accession nos of ESTs that showed higher expression in base					
AI385667.1	AK006407.1	AK006511.1	AK007900.1	AK009351.1	AK010774.1
AV256234.2	AV303305.1	AW049955.1	AW456706.1	BB045917.2	BB177770.2
BB432934.2	BB494451.1	BC022646.1	BQ175346.1	M55181.1	

are controlled by a large variety of neurotransmitters or a larger quantity than their apical counterparts, and that the mechanism of high-frequency sensitivity in the base is more complicated. Innervation patterns where hair cells in the base are more highly innervated than those in the apex [11] are consistent with this idea.

Among 39 known genes showing differential high expression in the apex, we detected only one ion transporter gene: Kcnj9. Kcnj9 is a member of the G protein-activated inward rectifier potassium channel family (GIRK/Kir3.x). GIRK generates slow inhibitory postsynaptic potentials following the activation of pertussis toxin-sensitive G protein-coupled receptors [12]. The spiral ganglion neurons isolated from the apical and basal regions of the cochlea showed differential firing features: low-frequency neurons from the apex displayed a high percentage of slowly accommodating neurons along with those that displayed prolonged action potential latency and duration [13]. GIRK3.3 might contribute to membrane conductance at negative potentials and, by extension, may contribute to the differences in latency observed between the apical and basal spiral ganglion neurons.

Among 60 known genes showing high differential expression in the base, seven genes were related to neurotransmission of genes. The number of genes relative to the neurotransmitter γ-aminobutyric acid (GABA) was particularly prominent. GABA is an inhibitory neurotransmitter, which is released from some efferent olivocochlear nerve endings terminating at outer hair cells. In this study, β2- and γ2-subunits of $GABA_A$ receptors were more highly expressed in the base than in the apex. However, the precise distribution of $GABA_A$ receptors remains controversial [14]. Previous reports have demonstrated that outer hair cells isolated from the apical cochlear turns did not respond to GABA during whole-cell recording [15]. The expression of β2- and γ2-subunits of $GABA_A$ receptors in the mammalian cochlea was independently validated by PCR [16]. ABR and DPOAE data from mice lacking β2-subunits of $GABA_A$ receptors demonstrated increasing threshold shifts from low to high frequencies [17]. Thus, our microarray analysis showing higher expression of β2-subunits of $GABA_A$ receptors is consistent with previous studies. In our microarray study, glutamic acid decarboxylase (GAD), which is required for the synthesis of GABA from glutamic acid, did not show differential expression in the apex or base. On the other hand, GABA transporter 2 and 3 expression was predominant in the base. This suggests that large amounts of GABA may be released from efferent auditory nerve terminals to bind to the cell of surface of outer hair cells in the base. The large amount of GABA-mediated hyperpolarization may contribute to fast adaptation of mechanotransducer current, which is characteristic of the high-frequency selectivity of hair cells [10] or the differential firing features of rapidly accommodating spiral ganglion neurons in the base [13].

The electrical resonant frequency of membrane potentials along the cochlear axis in mammalian hair cells has yet to be elucidated. Instead of this mechanism, isolated outer hair cells from the mammalian cochlea exhibit their unique mechanism of pre-amplification of sound by active somatic cell-body vibrations of outer hair cells [18]. By the gradient level of β2- and γ2-subunits as the component of $GABA_A$ receptors, outer hair cells may show the change in GABA-mediated OHC motility along the cochlear axis, and a subsequent modulation of the movement of the basilar membrane. This regulation may enhance the acute frequency selectivity and sensitivity of the mammalian cochlea.

Consistent with the physical properties of the basilar membrane described above, the receptor cells in the cochlea also show morphological gradations [19]. The stereocilia of the inner and outer hair cells of the mammalian cochlea vary systematically with location. Stereociliary length is maximal in the apex and decreases systematically along the length of

the cochlea, with the shortest stereocilia found in the basal cochlea. The hair cell soma, especially those of the outer hair cells, also changes in the same manner, with the largest cells present in the apex and the smallest in the base. In our analysis of differential expression pattern of genes that encode structural proteins, we were unable to measure a systematical difference between the apex and base. One possible setback was that our sample included both the organ of Corti and spiral ganglion neurons. While spiral ganglion neurons also vary systematically with cochlear location, the size–location relationship is opposite to that of the hair cells [20]. As such, this study did not detect any structural gene that would be predicted to have a major influence on the frequency selectivity.

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Otol Jpn 19(2):96-100, 2009　シンポジウム「血管条とラセン靱帯機能の新しい展開」特集

内耳蝸牛内高電位の成立機構

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Key words：endocochlear potential, hearing, ion transport, potassium (K^+)

はじめに

音は内耳蝸牛で受容される。蝸牛の有毛細胞の頂上膜に分布する感覚毛は、内リンパ液という特殊な細胞外液に触れている。これは、約150mMのK^+を含み、通常の細胞外液と同じイオン組成をもつ蝸牛の外リンパ液や血液に比べて約+80mVの高電位を帯びる（図1）。後者は蝸牛内高電位（EP：Endocochlear Potential）と呼ばれる[1]。内リンパ液のK^+は、音刺激により、感覚毛の頂部に局在する機械感受性チャネルを介して有毛細胞へ流入し、それを興奮させる。これが音伝達の第一ステップとなり音は中枢へ伝わる。有毛細胞の細胞体は、外リンパ液に浸され、静止膜電位は－60mVである。内リンパ液のEPは、有毛細胞へのK^+流入の駆動力を増大し、聴覚の音に対する高い感受性の成立に大きく貢献している。故に、内リンパ液が高電位・高K^+であることは、聴覚に必須である。

以前より、EPの成立は、蝸牛側壁の線維細胞群（螺旋靱帯）・上皮細胞群（血管条）を介した、内リンパ液から外リンパ液、そして内リンパ液へのK^+循環に立脚し、特に血管条がその中心的役割を果すとされてきた（図1）。血管条は、辺縁細胞からなる内層、中間細胞・基底細胞からなる外層、そして2層間を走る多数の血管から構成される（図1）。無酸素負荷や、血管条へNa^+、K^+-ATPase・Na^+、K^+、$2Cl^-$共輸送体・K^+チャネルの阻害薬を投与することにより、EPが著明に低下するため[2),3)]、K^+循環及びEPの成立には、蝸牛側壁に分布するこれらのK^+輸送装置が関与するとされてきた。組織学的実験により、上記ポンプと共輸送体は、辺縁細胞の基底側膜に発現することが報告された。しかし、K^+チャネルの分子実体や局在は不明であった。また、これらのK^+輸送装置が如何に共役してEPを成立させるかも謎であった。そこで、本研究はEPの成立機構の解明を目指した。

血管条の特徴とEP成立機構の仮説

血管条の解剖学的特徴として、①辺縁・基底・血管内皮細胞はタイトジャンクションで繋がれ、血管条内部を内・外リンパ液と血液から物理的に隔離する、②中間・基底細胞とその外側の線維細胞はギャップ結合で一体化され、合胞体を作る、③辺縁細胞の基底側膜と中間細胞の頂上膜はひだ状で互いに絡み合い、それらに挟まれた血管条細胞外空間（IS：Intrastrial Space）の間隔は15nmと狭い、という点が挙げられる[4)]。このISを満たす液は、通常の細胞外液と同じく低K^+（5 mM以下）であるが、約+90mVの高電位を示す（図1）[5),6)]。この電位はIntrastrial Potential：ISPと呼ばれ、EPの起源と考えられた[5)]。その後、❶Na^+、K^+、$2Cl^-$共輸送体・Na^+、K^+-ATPaseが辺縁細胞の基底側膜に強く発現すること[7)]、❷中間細胞を含む合胞体の電位は約－5 mVであること、が報告された[5)]。故に、①❶のK^+輸送装置によって保たれていると予想されるISの低K^+と合胞体内との間に大きなK^+の濃度差があるので、②K^+チャネルが中間細胞若しくは基底細胞の頂上膜に存在すると仮定すると、③合胞体頂上膜を介して90mV以上のK^+拡散電位が生ずる。そしてこの電位が、中間細胞が－5 mVであるため（上記❷）、そのまま+90mVのISPとなり、更に辺縁細胞で約10mV降下して（成因は不明）、最終的に+80mVのEPを示すという仮説がSaltらにより発表

された[5]（図1）。

EP成立のための必須分子Kir4.1の同定

まず、K^+チャネル阻害薬であるBa^{2+}の標的分子の同定を試みた。最初に生理実験によってBa^{2+}の作用を確認し、また、電位依存性K^+チャネルの阻害薬はEPに殆ど影響しないことを見出した。よって、EP成立には、もう一つのチャネルファミリーである内向き整流性K^+（Kir）チャネルが重要であることが判明した。15種類のKirサブユニットの中で、RT-PCR法によりKir4.1のみが血管条に発現していることを見出した。免疫組織実験にて、血管条におけるKir4.1の強い分布を確認した。以上より、Kir4.1はEP成立の必須分子であると考えられた[8]。後に竹内らによって、Kir4.1は中間細胞の頂上膜に局在することが報告され、このチャネルはISPの責任分子であることが示唆された[9),10]。近年、Kir4.1欠損マウスは、EPが欠如し、高度難聴を示すことが観察された[11]。

K^+輸送装置阻害下におけるIS環境とEPの変化

上記の通り、EP成立に重要な分子が同定されて来たが、Saltらの仮説の実験的な証明は、阻害薬を還流しつつ15nmと狭いISや合胞体に電極を留置することが困難なため、行われなかった。また、動物を無酸素に曝すと、EPは−10mVまで減少するが、血管条の内部は+14mVまでしか低下せず[12]、これはISPとEPがほぼ同等のものとする従来の仮説のみでは説明できない。そこで、仮説の妥当性を検証し、EP成立に必要な他の要素を見出すため、K^+選択的イオン電極（K^+電極）をモルモットの蝸牛側壁に挿入し、血管条の細胞内外の微小区域のK^+濃度・電位を種々の条件下で測定した。同時に別の電極でEPの変化も観察した[13]。

図2A上段は、K^+電極を外リンパ液から螺旋靭帯・血管条・内リンパ液へと進めた際の、電位（赤）とK^+濃度（青：活量で示す）の変化を示し、下段は、通常の電極で内リンパ液の電位、即ちEPを経時的に観察したものである。また、血管条に薬剤を投与するため、血管条毛細血管の源流である椎骨動脈にカテーテルを留置した。外リンパ液（0mV・低K^+）よりK^+電極を進めると、最初にK^+濃度が65～85mMと高く、電位が+2～3mVと軽度正の値を示す地点（黒矢印）を見出した。解剖学的に（図1）、これは中間・基底・線維細胞から成る合胞体の内部を観察していると考えられた。更に電極を進めると、電位が+70mVと高く、K^+濃度が4mMと低いISを認めた（白矢印）。電極を留置し、無酸素負荷により血管条のNa^+、K^+-ATPaseを阻害したところ、ISの電位（ISP）は+70mVから+22mVへと下降して3mVだけ上昇し、K^+濃度は4mMから28mMへと上昇して2mMのみ下降する、二層性変化を認めた（図2A点線四角と図2C）。同時にEPは−14mVの負の値まで下降し（図2A下段）、正の値で留まったISPとの差が認められた（白矢頭）。無酸素を解除すると、全ての値は元に戻った。K^+電極をISに留めたまま、次にK^+チャネル阻害薬Ba^{2+}を血管条へ投与した。ISPは+23mVまで減少し、同時にEPの低下も認めたが、無酸素負荷の場合と異なり、ISのK^+濃度は殆ど変わらなかった（図2A黒矢頭）。

これらの結果、特に無酸素負荷時のISPとISのK^+濃度の鏡像反応は、中間細胞頂上膜のBa^{2+}感受性K^+チャネルKir4.1を介したK^+拡散電位がISPの主要素であることを示唆する。一方、中間細胞にはCl^-やNa^+の電流が殆ど認められないので[14]、ISPは、ネルンストの式より以下のように簡略化した式で求められる。

$$ISP = V_{Syn} + \frac{RT}{F}\ln\left[\frac{aK^+_{i(Syn)}}{aK^+_{IS}}\right] \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (a)$$

V_{Syn}は合胞体の電位、$aK^+_{i(Syn)}$とaK^+_{IS}は合胞体とISのK^+濃度である。また、合胞体の電位とK^+濃度は、無酸素負荷によって殆ど影響されなかった（データ示さず）。よって、V_{Syn}と$aK^+_{i(Syn)}$は一定で、図2A上段の実測値（黒矢印）を代入した。更に（a）の式へ、無酸素時におけるISのK^+濃度変化の実測値（図2A上段点線四角と図2B下段青）を代入して、ISPの予想値（図2B下段緑）を求め、ISPの実測値（赤）と比較した。両者はよく一致した（図2B上段）。故に、ISPは主に中間細胞のKir4.1を介したK^+拡散電位により成立することが実証された。

辺縁細胞の役割

図2Aの無酸素負荷時に認められたISP値とEP値との差（白矢印）から、EPの成立にはISP以外の要素が必要であると予想された。この電位差は、解剖学的にISと内リンパ液の間にある辺縁細胞で生じていると予想されたので、具体的な部位の同定を試みた[13]。K^+電極を外リンパ液から進めていくと（図3A上段）、僅かに正電位かつ高K^+濃度を示す合胞体（黒矢印）、高電位かつ低K^+濃度を示すIS（黒矢頭）を認め、その後、高電位

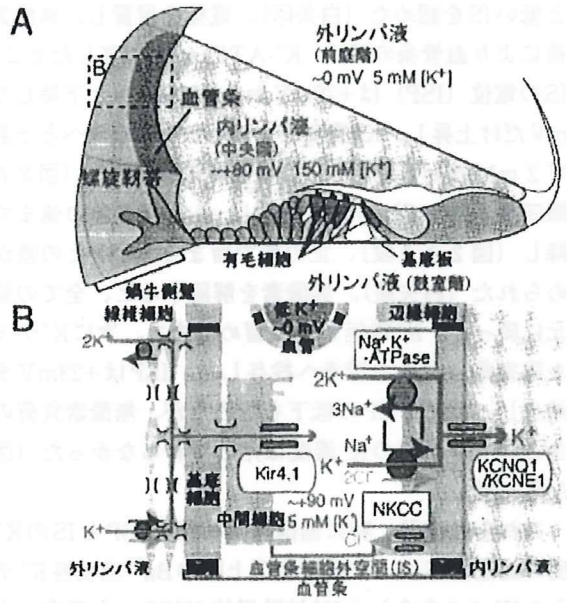

図1　内耳蝸牛（A）と側壁（B）の構造とイオン輸送装置

NKCC：Na^+, K^+, $2Cl^-$ 共輸送体, TJ：タイトジャンクション
図1～3は全て文献（13）より改変して引用。

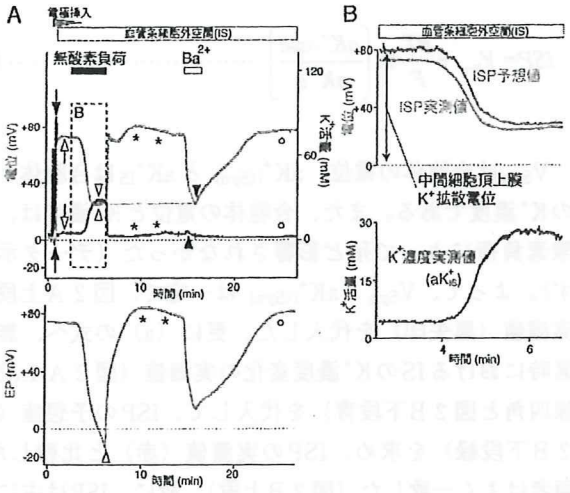

図2　Na^+, K^+-ATPaseとK^+チャネル阻害の効果

（A）赤は電位を、青はK^+濃度（活量）の変化を示す。無酸素負荷（黒棒）及びウアバインの還流（赤棒）でNa^+, K^+-ATPaseを、Ba^{2+}の還流（黄棒）でK^+チャネルを阻害した。上段：K^+電極による蝸牛側壁のK^+濃度と電位の測定。下段：別電極によるEPの経時変化。（B）無酸素下でのISのK+濃度の変化（Aの点線四角のデータ）からISPの予想値（緑）を求め、ISPの実測値（赤：A点線四角の赤線と同じ）と比較した。EL：内リンパ液

のままでK^+濃度が急に約80mMと高くなる点を見出した（白矢印）。内リンパ液のK^+濃度は100mM以上であるため、この場所は辺縁細胞の内部であると考えられた。電極を留置し、無酸素を負荷したところ、電位は+74mVから+27mVへ大きく下降した後、+52mVへ回

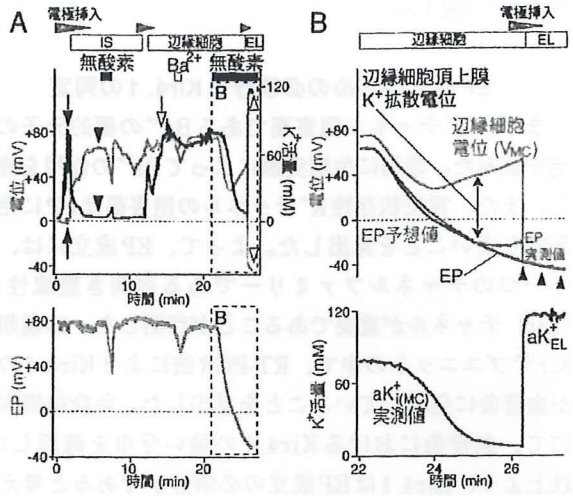

図3　辺縁細胞の解析

（A）上段：K^+電極による測定。合胞体（黒矢印）、IS（黒矢頭）を通過後、高電位・高K^+の辺縁細胞内（白矢印）に到達。無酸素負荷の後、内リンパ液へ挿入した。下段：EPの変化。（B）無酸素によるK^+濃度の実測値（下段青：Aの点線四角と同じ）から辺縁細胞頂上膜のK^+拡散電位を求め、辺縁細胞電位の実測値との和をEPの予想値（緑）として示した。更にEPの実測値（灰色：A下段点線四角）を重ねた。EL：内リンパ液

復したが、K^+濃度はS字状に低下した（図3A上段点線四角）。この辺縁細胞の電位変化は、無酸素時のISPの二層性変化に類似している（図2A）。一方、EPは低下し続け、−40mVを示した。更にK^+電極を辺縁細胞内から内リンパ液へと進めると、K^+濃度は約100mMへ上昇し、電位は大きく下降してEPとほぼ同じ負値を示した（図3A白矢頭）。従って、図2Aで観察されたISPとEPの差は、辺縁細胞の頂上膜で生じていたことになる。

辺縁細胞の基底側膜にはK^+電流が認められないが、頂上膜には電位依存性K^+チャネルKCNQ1/KCNE1が分布する（図1B）。故に、図3A上段のように辺縁細胞内のK^+濃度が低下すると、頂上膜におけるK^+拡散電位が増大し、EPと辺縁細胞との電位差が拡大する。辺縁細胞頂上膜のK^+透過性はNa^+やCl^-よりもかなり大きいため[15]、EPは単純に辺縁細胞の電位と頂上膜のK^+平衡（拡散）電位の和として計算できる。

$$EP = V_{MC} + \frac{RT}{F}\ln\left(\frac{aK^+_{i(MC)}}{aK^+_{EL}}\right) \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (b)$$

V_{MC}・aK^+_{MC}はそれぞれ辺縁細胞の電位・K^+濃度である。内リンパ液のK^+濃度（aK^+_{EL}）は無酸素負荷により変化しないため[16]、一定である。(b) 式へ、無酸素による辺縁細胞の電位・K^+濃度変化の実測値（図3A上

段点線四角）を代入し、EPの予想値を求めた（図3B上段の緑）。この値は、EP変化の実測値（図3A下段点線四角と図3B灰色線）とよく合致した。従って、無酸素負荷時に拡大する辺縁細胞頂上膜を介した電位差は、主にK^+拡散によることが明らかとなった。

血管条の電気的隔絶

ISPを高く保つため、ISは隣接する内・外リンパ液や血液から電気的に絶縁されているという仮説を立てた。その証明のため、K^+電極に通電することで、組織の入力抵抗を測定する実験を試みた（データ示さず）[13]。内リンパ液は外リンパ液と同レベルの低い抵抗値を示したが、ISでは高い抵抗値が観察された。即ちISは、隣接する細胞外液から電気的に隔離されていることが判明した。

結果の総括

本研究において、EP成立の必須分子Kir4.1を同定した。また、血管条内のISの存在と特異的な環境（高電位・低K^+）を確認した。更に、EPの主な起源がISPであること、中間細胞頂上膜を介した電位差は大部分がK^+拡散電位であり且つISPの主成因であることを示し（図2）、Saltらが提唱した仮説[5]をはじめて実証した。

また、新たにEP成立に必要な3要素を同定した。第一は、ISが隣接する外リンパ液・内リンパ液・血液から電気的に隔絶されていることである。これはISが+90mVの高電位ISPを維持するために不可欠である。タイトジャンクションで繋がった基底・辺縁・血管内皮細胞層の3層がそれぞれ絶縁機能を有すると予想される（図1）。第二は、中間細胞を含む合胞体の電位が固定されていることである。図2A及び別個体の検討によって、この電位は過去の報告[5]と異なり、軽度の正値を示すことが判明した。この特性のため、中間細胞の頂上膜を介したK^+拡散電位が、高電位のISPとして反映される。

第三は、辺縁細胞頂上膜を介したK^+拡散である。辺縁細胞のNa^+、K^+-ATPaseを阻害した際の、ISPとEPの間の差（図2）は、辺縁細胞内のK^+濃度の低下によって拡大する辺縁細胞頂上膜のK^+拡散電位によって説明できた（図3）。また辺縁細胞の基底側膜にはK^+チャネルが殆どないため[17]、辺縁細胞の電位はISのK^+濃度変化の影響を受けず、ほぼISPと同値と考えられる。故に、EPは、(a)（b）より以下の式で求められる。

$$EP = V_{Syn} + \frac{RT}{F}\ln\left[\frac{aK^+_{i(Syn)}}{aK^+_{IS}}\right] + \frac{RT}{F}\ln\left[\frac{aK^+_{i(MC)}}{aK^+_{EL}}\right] \quad \cdots\cdots (c)$$

通常、辺縁細胞と内リンパ液のK^+濃度は、各々約80mMと100mM以上であるため、辺縁細胞の電位とEPとの約10mVの差は、主にK^+拡散電位によるものと考えられる。

おわりに

本研究により、EPの成立機構は定性的にほぼ理解できたと考えられる。今後は、実験や計算科学の手法を用いて、定量的により正確に解明することを目標としたい。

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