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Measurements of ionic concentrations along with endocochlear potential in wild-type and *claudin 14* knockout mice

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ABSTRACT

Objective: To examine whether the changes in endolymphatic ion concentrations were involved in hair cells degeneration in *claudin-14* knockout (KO) mice (*Cldn14*$^{-/-}$), we measured the endocochlear potential (EP) along with concentrations of K^+, Na^+, H^+, or Ca^{2+} ($[K]_e$, $[Na]_e$, pH_e, $[Ca]_e$) in *Cldn14*$^{-/-}$, in which hair cells were selectively damaged, and compared with measurements in wild type mice (Wt).

Methods: We used the *Cldn14*$^{-/-}$ from 3 weeks of age, in which the auditory brain responses (ABR) was severely diminished. Using double-barreled ion-selective microelectrodes, we measured $[K]_e$, $[Na]_e$, pH_e, and $[Ca]_e$ in both Wt and *Cldn14*$^{-/-}$ at 8–10 weeks of age.

Results: (1) In Wt, the EP was +92 mV. $[K]_e$, $[Na]_e$, pH_e, and $[Ca]_e$ were 169 mM, ~1.0 mM, 7.50, and 395 nM, respectively. In the *Cldn14*$^{-/-}$, the EP was +96 mV. $[K]_e$, $[Na]_e$, pH_e, and $[Ca]_e$ were 167 mM, ~1.0 mM, 7.73, and 179 nM, respectively. No significant differences in the above values were observed between Wt and *Cldn14*$^{-/-}$. (2) A significant linear correlation between EP and $[Ca]_e$ ($R = 0.93$) was observed for both Wt and *Cldn14*$^{-/-}$, but no correlation was observed between EP and K^+, Na^+, or H^+.

Conclusion: These findings suggest that (1) the changes in endolymphatic ion concentrations might not be involved in hair cells degeneration in *Cldn14*$^{-/-}$, (2) $[Ca]_e$ might be regulated by EP in both Wt and *Cldn14*$^{-/-}$.

1. Introduction

The relationship between deafness and a mutation in the gene encoding the tight junction (TJ), claudin 14, was first implicated from the analysis of human families. *In situ* hybridization and immunohistology demonstrated the expression of claudin 14 in the sensory epithelium of organ of Corti [1]. The *claudin-14* knockout (KO) mice (*Cldn14*$^{-/-}$) were deaf, as was revealed by auditory brain response (ABR) at 4 or 12 weeks of age. The defect was caused by the degeneration of cochlear hair cells. Interestingly, the change of endocochlear potential (EP) was not observed in KO mice, suggesting that the degeneration of cochlear hair cells might be induced by a change in the ion composition of Nuel's space [2]. In an independent study, Boettger et al. reported the involvement of the K^+ transport in deafness, especially the K-Cl co-transporter (Kcc4) in supporting hair cells (Deiter's cell) [3]. They also demonstrated that the loss of K-Cl co-transporter (Kcc3) slowly produced deafness, induced by the degeneration of hair cells without the change in EP and endocochlear K^+ concentration

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($[K]_e$). Based on these findings, they suggested that the change of local K^+ concentration in space surrounding hair cells may produce deafness [4].

Other groups also reported that the loss of claudin 9 or tricellulin produced deafness without a change in EP, probably due to the changes in local K^+ concentration around hair cells or in permeability for K^+, Na^+, or ATP across the TJ between endolymph and perilymph [5,6,7]. Recently, Kitajiri and Katsuno published a review discussing the relationship between the deafness and the tricellular tight junctions in the inner ear [8]. Interestingly, they indicated that the hair cell damage induced by the loss of the claudin 9, claudin 14, occuldin, angulin-2/IRDR1, or tricellulin overlaps with the "rapid phase of EP formation" and suggested that the difference of timing for the initiation of hair cell degeneration might be caused by the differences in paracellular permeability associated with each of the TJ proteins.

In the current study, we independently generated *Cldn14*$^{-/-}$ and examined the change of permeability across the tight junction, measuring the EP and the ion concentrations, such as K^+, Na^+, and H^+ ($[K]_e$, $[Na]_e$, and pH_e). Recently, Thomas and Bers successfully fabricated the double-barreled PVC-resin Ca^{2+}-selective microelectrodes and reported that these electrodes were more useful than fura-2 for the measurement of Ca^{2+} within the range of 10^{-6}–10^{-8} M in snail neurons [9,10]. Therefore, we also measured Ca^{2+} concentration in endolymph ($[Ca]_e$) in this study.

The present results demonstrated that (1) no significant difference in the EP, $[Ca]_e$, $[K]_e$, $[Na]_e$, and pH_e was observed between wild type mice (Wt) and *Cldn14*$^{-/-}$, indicating that the degeneration of hair cells was not induced by these changes, (2) $[Ca]_e$ was low (~0.3 μM) in both Wt and *Cldn14*$^{-/-}$ at ~+85 mV of the EP, (3) in both Wt and *Cldn14*$^{-/-}$, correlation between EP and $p[Ca]_e$ (=−log $[Ca]_e$) was observed (R = 0.93), but no correlation was observed between EP and $[K]_e$, $[Na]_e$, or pH_e.

2. Materials and methods

2.1. Fabrication of H^+-, K^+-, Na^+-, Ca^{2+}-selective microelectrodes

Single-barreled H^+-, K^+-, Na^+-, or Ca^{2+}-selective microelectrodes or double-barreled H^+-, K^+-, or Na^+-selective microelectrodes were fabricated by the method reported earlier [11–16]. In particular, calibration for Na^+-selective microelectrodes was performed in 1 mM, 3 mM, 5 mM NaCl, and 10 mM NaCl + 150 mM KCl solution before and after impalement into endolymph. After experiments, tissue surface of cochlea was perfused with 10 mM NaCl + 150 mM KCl solution to calibrate Na^+-selective microelectrodes.

Double-barreled PVC-resin Ca^{2+}-selective microelectrodes were fabricated as reported previously with slight modifications [9,10]. Briefly, (1) Double-barreled pipettes with one end of the capillary (for use as the reference electrode) closed by a fine flame to protect against silanization were prepared. (2) After the silanization, the tip of double-barreled pipettes were ground by a glass grinder (EG-44, Narishige, Tokyo, Japan), producing a tip size of 2–3 μm by monitoring the capillary phenomenon under a stereomicroscope (Model ES7, Olympus, Tokyo). Subsequently, the PVC-containing Ca^{2+}-selective ion-exchanger ETH-1001 (PVC-resin), prepared as previously described [13,14], was injected from the end of the ion-selective barrel with a fine polyethylene tube, up to the shoulder of the pipette.

After filling the pipette with the PVC-resin, micropipettes with PVC-resin were stored in a refrigerator [14]. The internal solution for the Ca^{2+}-microelectrodes, introduced immediately before use, was 10^{-7} M Ca^{2+} solution containing 145 mM K^+. The reference barrel was filled with 0.5 M KCl solution.

When we advanced the single-barreled ion-selective microelectrode from the tissue surface of cochlea into the endolymph, the voltage deflection to the positive potential was observed consistently, and the observed potential remained stable over 1 min. After measuring the voltage for endolymphatic ion concentration plus EP by using the single-barreled ion-selective microelectrode, the EP was measured again with a conventional microelectrode by its insertion through the same pore punctured by the ion-selective microelectrode. Subsequently, the value of the voltage change with the ion-selective microelectrode was subtracted from the EP to obtain the endolymphatic ion concentration.

Ion-selective microelectrodes were calibrated before and after experiments. The criteria for use of the obtained data were described previously [13–16].

2.2. Generation of *Cldn14*$^{-/-}$

Cldn14$^{-/-}$ mice (Accession No. CDB1000K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) were newly generated [2]. *Cldn14*, the gene encoding claudin 14, has three exons, with the entire protein of 239 amino acid residues encoded in the third exon. The targeting vector were constructed (Fig. 1A) and used to replace the exon 3 with the neomycin resistant gene by homologous recombination in HK3i ES cells [17].

Two lines of mice were generated from distinct recombinant ES cell clones, in which one of the two wild type *Cldn14* alleles was disrupted (*Cldn14*$^{+/-}$, Fig. 1B). Each genotype was confirmed by PCR analysis (performed by Unitech, Chiba, Japan; Fig. 1C). The primer sequences were: TGACAGAAA-TAAAGGCATCGAGATA (forward); GTGAGCGGTAGAT-CTGACACTGGTA (reverse 1); CGTGCAATCCATCTTGT-TCAAT (reverse 2).

2.3. RNA analyses

Total RNA was extracted using TriPure Isolation Reagent (Roche). Reverse transcription was performed on 0.5–1 μg of total RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems). PCR amplification was performed as described previously [2] and specific bands of cldn14 were detected only in the Wt lane (Fig. 1D).

2.4. ABR measurement in Wt and *Cldn14*$^{-/-}$

Hearing was evaluated by ABR analysis and 3-4 mice were tested at 3, 6 and 9 weeks of age in both Wt and *Cldn14*$^{-/-}$. All

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