Evidence and main site of ATP secretion in the vestibular system by mechanical stimulation

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Directed by Professor Sung Huhn Kim

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Hyun Jin Lee

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This certifies that the Doctoral Dissertation of Hyun Jin Lee is approved.

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ABSTRACT

Evidence and main site of ATP secretion in the vestibular system by mechanical stimulation

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The vestibular system is important in maintaining balance during acceleration stimulation in daily life. The cochlear perilymph and endolymph have long been known to contain nanomolar concentrations of ATP that can mediate cochlear and hearing functions through various purinergic receptors. Similarly, ATP is thought to exist in vestibular organs to modulate the function of balance system; however, direct functional evidence for the ATP release in the vestibular labyrinth is lacking. The goal of the present study was to investigate the evidence and location of ATP release in vestibular organs. The membranous vestibular labyrinths of C57BL/6 mice were harvested and the utricle, saccule, ampulla, and common crus were separated by microdissection. To measure the ATP secretion rate per area of each tissue, the surface area of each tissue was calculated by the geometric method with a confocal microscope. Rotational mechanical stimulation was applied with a three-dimensional multi-shaker. The amount of ATP secretion was measured by a bioluminescent-based method using an EDTA-containing luciferin-luciferase assay kit. In selected samples, bafilomycin A1 and carbenoxolone (CBX) were applied to inhibit ATP secretion by vestibular dark cells and connexin hemichannels. ATP-enriched vesicles

were stained with quinacrine for the localization of ATP secretion. We investigated the distribution of connexin 26 (CX26) in mouse vestibular dark cell areas by immunohistochemical staining. The ATP secretion per surface was significantly increased during the stimulation (0.11 ± 0.03 nM/mm^2, 0.44 ± 0.18 nM/mm^2, and 0.05 ± 0.01 nM/mm^2 in utricle with ampulla, saccule, and common crus, respectively). The amount of ATP secretion in saccule was significantly higher than that in other vestibular organs ($p < 0.05$). ATP secretion was partially or completely blocked by bafilomycin A1 (100 nM) but was almost completely blocked by CBX (10 nM) in all tissues. Expression of the ATP containing vesicle was detected in the dark cell area in the utricular roof epithelium, utricular macula, around the ampullary crest, and in the common crus and saccular macula. CX26 was identified to be consistently distributed within the vestibular dark cell area in the roof epithelium and common crus. This is the first study to demonstrate that mechanical stimulation induces ATP secretion in the vestibular organs. The main sites of ATP secretion are thought to be the macular gap junction hemichannels and dark cell areas. The results of this study justify the role of ATP mediated ion transport via purinergic receptors for various physiological responses in the vestibular system during mechanical stimulation.

Key words: ATP, vestibule, inner ear, mechanical stimulation, dark cell

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I. INTRODUCTION

Adenosine triphosphate (ATP) is a key signaling molecule in the inner ear. In the cochlea, ATP is secreted by mechanical stimulation (sound exposure) in a paracrine manner.[1] ATP regulates sound transduction, hearing sensitivity, active mechanical amplification by outer hair cells, cochlear potential, cochlear homeostasis, and vascular tension via purinergic receptors distributed over sensory and extra-sensory epithelial cells of the cochlea.[2-4] ATP has been reported to be secreted through the hemichannel of gap junctions at supporting cells and as a vesicular release at marginal cells of the stria vascularis.[5-7]

The vestibular system plays an important role in maintaining balance by providing stable vision and posture during movement. The vestibule is situated in the temporal bone of the skull and comprises the saccule, utricle, and three semicircular canals with ampulla; the vestibule contains various types of epithelial cells that have similar molecular and functional identities to those of cochlear epithelial cells. For the sensory epithelium, type 1 and type 2 hair cells correspond to cochlear inner and outer hair cells and detect mechanical

stimulation such as angular and linear acceleration.[8-10] Supporting cells are punctuated among vestibular hair cells and have gap junctions between them. Dark cells are extra-sensory epithelial cells of the vestibule and have similar functional roles and molecular components to cochlear strial marginal cells.[11,12] The main role of vestibular dark cells is secretion of K^+ through KCNQ1 protein channels into the luminal space of vestibule as cochlear strial marginal cells.[13,14] Considering the functional and molecular similarities between the vestibule and cochlea, ATP is likely to have a similar role in the regulation of ion homeostasis and vestibular function compared with that in the cochlea. Evidence of this is demonstrated by the presence of various purinergic receptor found in sensory and extra-sensory epithelial cells in the vestibular labyrinth.[15,16] However, there has been no direct evidence for ATP secretion or the site of secretion by mechanical stimulation in the vestibular system.

We hypothesized that the vestibular organs possess a specific ATP secretion system. In this study, we investigated the secretion and source of this ATP in the vestibular system by measuring ATP secretion after mechanical stimulation *ex vivo* and by quinacrine staining. The results of this study provide basic information for understanding vestibular physiology and ion homeostasis in the vestibular system and the complex mechanisms used to maintain balance.

II. METHODS

1. Dissection of vestibular organ from mouse

Male and female C57BL/6 mice aged 8 to 12 weeks (Orient Bio, Seoul, Republic of Korea) were used for the experiment. Mice were anesthetized with

tribromoethanol (0.016 mg/g, intraperitoneal; Sigma-Aldrich, St. Louis, USA) and the temporal bone was freshly isolated after decapitation. The cochlear was removed from the temporal bone, and the bony shell of the vestibular organ was carefully peeled-off using micro-forceps and needles in a sterile perilymph-like solution (150 mM NaCl, 3.6 mM KCl, 1mM $MgCl_2$, 0.7 mM $CaCl_2$, 10 mM HEPES, 5 mM dextrose, pH 7.4). The membranous labyrinth of vestibular organ was micro-dissected and harvested. Finally, the utricle, saccule, ampulla, and common crus were completely exposed (Fig 1). The vestibular organ was divided into three pieces for testing ATP release in each subdivision: utricle with ampulla, saccule, and common crus. All experimental procedures were performed at room temperature.

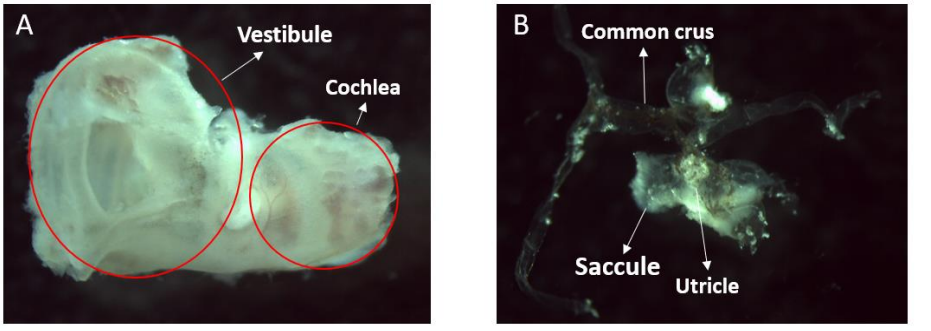

Figure 1. Dissection of the vestibular organ. (A) Harvested temporal bone. (B) Membranous vestibular labyrinth after micro-dissection.

2. Ethics statement

Institutional Animal Care and Use Committee of Yonsei University College of

Medicine approved this study, and all mice were treated in accordance with the guidelines for the Care and Use of Laboratory Animals of Yonsei University College of Medicine (approval No.2016-0254).

3. Surface area measurement of vestibular organ

To measuring the ATP secretion rate per area of each tissue, the surface area of each tissue was calculated immediately after the preparation of the tissue. Each tissue was placed on a glass-bottom dish containing perilymph-like solution and z-stack images at different 90-degree angles were obtained with a confocal microscope (LSM780, Carl Zeiss, Jena, Germany). First, an axial image of the tissue was obtained, and then the tissue was rotated 90 degrees using three-axis hanging Joystick oil hydraulic micromanipulator (Narishige, Minamikarasuyama 4-chome, Setagaya-ku, Tokyo, Japan) and a further image was obtained. The irregular shape of the tissues of the vestibular organ required the internal surface area to be calculated assuming that the utricle and saccule were elliptical shaped and that the common crus was cylindrical shaped. The internal surface area of each tissue was calculated as follows.

First, we measured the largest luminal area in the z-stack image by drawing the line of the inner border of each luminal area with ZEN software (Carl Zeiss) (Fig. 2A). The formula for obtaining the distance of x-axis and z-axis of elliptical shape (utricle with ampulla and saccule) was implemented in the following equation:

$$D\ (\mu m) = 2\times \sqrt{\frac{A}{\pi}}$$

Where *D* is the distance of x-axis and z-axis of elliptical shape; and *A* is the measured area of elliptical shape (Fig. 3).

The formula for obtaining the surface area of ellipsoid was implemented using Knud Thomsen's formula where the error ratio is approximately 1.061%:

$$S\ (mm^2) = 4\times \pi \times \left\{\frac{\left[\left(\frac{D1}{2}\right)\times\left(\frac{D1}{2}\right)\right]^{1.6}+\left[\left(\frac{D1}{2}\right)\times\left(\frac{D2}{2}\right)\right]^{1.6}+\left[\left(\frac{D1}{2}\right)\times\left(\frac{D2}{2}\right)\right]^{1.6}}{3}\right\}^{1/1.6} \times 10^{-6}$$

Where *S* is the surface area of utricle and saccule; *D*1 is the x-axis distance of elliptical shape; and *D*2 is the z-axis distance of elliptical shape.

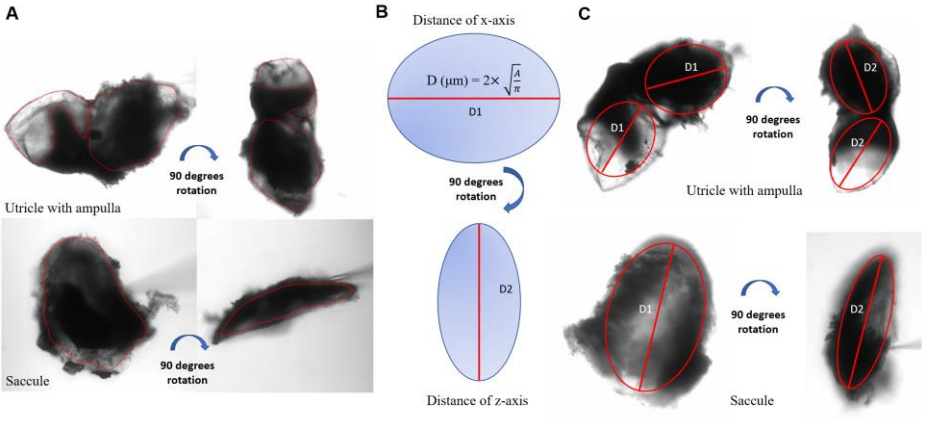

Figure 2. Schematic diagram and images of measuring ellipsoid surface area. The z-stack images of different 90-degree angles were obtained with a confocal microscope (LSM780, Carl Zeiss, Jena, Germany). (A) The z-stack image by drawing the line of the inner border of each luminal area with ZEN software (Carl Zeiss). The x-axis and z-axis of utricle with ampulla and saccule.

(B) Schematic diagram for obtaining the distance of x-axis and z-axis of elliptical shape and the formula of the distance. (C) The images of different 90-degree angles were obtained with a confocal microscope in the utricle with ampulla and saccule. *A*, area of elliptical shape of utricle and ampulla*; D1, 2*, the distance of x-axis and z-axis of elliptical shape.

The surface area of common crus was obtained with the formula

$Sc\ (\mathrm{mm}^2) = 2\,\pi\,r\,h\ \times 10^{-6}$

Where *Sc* is the surface area of common crus; *r* is the radius of the bottom circle of common crus; *h* is the eight of the common crus (Fig. 3):

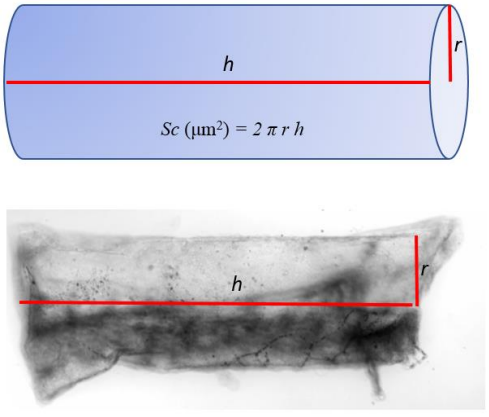

Figure 3. Schematic diagram and image of measuring cylindrical surface area. *Sc*, surface area of common crus; *r*, radius of the bottom circle of common crus; *h*, height of the common crus.

4. Application of mechanical stimulation

Mechanical stimulation was applied by rotational stimuli using three-dimensional (3D) 300 shaker (FINEPCR, Gyeonggi-do, Republic of Korea). Each dissected tissue was transferred to 48-well culture dishes containing perilymph-like solution (200 μL). A consistent mechanical stimulation at 250 rpm was applied on the shaker (15 min, three times). After each stimulation, the ATP secretion level was measured; the tissue was then carefully washed three times with perilymph-like solution, and the bath solution was refreshed.

5. ATP concentration measurement

The amount of ATP secretion was measured by a bioluminescent-based method with an EDTA-containing luciferin-luciferase assay kit (Invitrogen, Carlsbad, USA). The bioluminescence was read by a luminometer (Centro XS^3 LB 960, Bad Wildbad, Germany) using a black 96-well plate to avoid optical cross-talk. Bath solution (200 μL) was sampled after each stimulation and testing reagent was added prior to measurement. The amount of ATP was calculated from the ATP standard curve, which is simultaneously measured from a serially 10-fold diluted ATP standard in each experiment. The background luminescence was obtained by adding the same volume of perilymph-like solution. The standard curve served as an internal control for machine measurement, which had good linearity. The change in ATP concentration was calculated with respect to the initial resting level (ΔATP).

6. Inhibitor study for ATP secretion

To investigate the physiological mechanisms for ATP release, bafilomycin A1 (B1793, Sigma-Aldrich) and carbenoxolone (CBX) (C4790, Sigma-Aldrich) were used. Bafilomycin inhibits vacuolar H^+-ATPase[7,17] and was used to investigate vesicular release of ATP from dark cells, whereas CBX inhibits connexin hemichannels and was used to investigate the release of ATP through these channels.[18,19] Bafilomycin (100 nM) or CBX (10 nM) was added to the bath solution, and then mechanical stimulation was applied with the same protocol described above; this was followed by measurement of ATP concentration.

7. Staining of ATP-containing vesicle using quinacrine

Quinacrine (Sigma-Aldrich), an acridine derivative, has a very high affinity for ATP and has been used to label intracellular ATP-enriched vesicles in neuronal and epithelial cell types.[20] We used quinacrine to identify the location of ATP secretion. Vestibular membranous labyrinths were prepared as described above. The prepared tissue was placed in glass-bottom dishes containing phosphate-buffered saline (PBS) (137 mM NaCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO4, and 2.7 mM KCl, pH 7.4). Isolated vestibular organs in the glass-bottom dishes were stained with quinacrine dihydrochloride (5×10^{-6} mol/L; 1 × PBS) for 3 min at room temperature in the dark. After staining, the tissues were washed seven times with PBS. Finally, the tissues were placed on a slide and a cover-slip added with mounting medium (FluorSave, 345789, EMD-Millipore, Darmstadt, Germany). Stained images were obtained with a

confocal microscope (LSM780).

8. Immunohistochemistry

The prepared whole-mounted tissues were permeabilized and blocked in phosphate-buffered saline (PBS) with 0.15% Triton X-100 (PBS-TX) and 5% bovine serum albumin (BSA) at room temperature. These were then washed three times with PBS-TX and incubated overnight at 4°C with polyclonal antibodies against connexin 26 (71-0500, Invitrogen) in PBS-TX with 2.5% BSA. Then, the tissues were washed five times with PBS-TX and incubated with Alexa-568-rabbit anti-mouse antibody (1:1000, A11004, Invitrogen), FITC-conjugated phalloidin antibody (1:200, P5282, Sigma-Aldrich), and 4′,6-diamidino-2-phenylindole (DAPI) (1:10000, D1306, Thermo Fisher Scientific) in PBS-TX with 2.5% BSA at room temperature for 1 h. The tissues were washed five times with PBS-TX and mounted between two coverslips with Fluoromount™ Aqueous Mounting Medium (F4680, Sigma-Aldrich). The prepared tissues were imaged using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

9. Data analysis and statistics

In each dataset, normality and equal variance were evaluated by the Shapiro–Wilk test and Brown–Forsythe test. The effect of mechanical stimulation was investigated in independent experiments and the significance of the ATP secretion was evaluated by the t-test or Mann–Whitney Rank Sum test. Differences of the ATP secretion in each tissue after mechanical stimulation and

in the effects of inhibition test in three different tissues were evaluated by one-way ANOVA with Holm–Sidak's post-test or Kruskal–Wallis one-way ANOVA on ranks with Dunn's post-test. Statistical analysis was performed with SigmaStat (Systat Software, San Jose, USA). Results are presented as mean ± SD for samples. $p < 0.05$ was considered significant.

III. RESULTS

1. ATP secretion in vestibular organ

To measure the amount of ATP secretion per unit area, the unit area of each tissue was calculated first. We calculated the surface area by separating tissue into utricle with ampulla, saccule, and common crus. The measured surface area of each organ was: utricle and ampulla, 5.13 ± 0.34 mm^2; saccule, 2.32 ± 0.41 mm^2; and common crus, 2.67 ± 0.47 mm^2.

We investigated the secretion of ATP in the vestibular organ by mechanical stimulation. In the utricle with ampulla, the amount of ATP secretion per surface area was initially 5.02 ± 0.39 nM/mm^2 and increased with a ΔATP of 0.11 ± 0.03 nM/mm^2 with the first mechanical stimulation. The ΔATP secretion per surface area in the first mechanical stimulation was significantly higher compared with that of the initial resting, second mechanical stimulation, third mechanical stimulation, and final resting state ($p < 0.001$) (Fig. 4A, D). In the saccule, the amount of ATP secretion per surface area was 11.79 ± 1.52 nM/mm^2, and this also increased in comparison with the first mechanical stimulation with a ΔATP of 0.44 ± 0.18 nM/mm^2. The ΔATP secretion per

surface in the first mechanical stimulation was also significantly higher compared with that of the initial resting, second mechanical stimulation, third mechanical stimulation, and final resting state ($p < 0.005$) (Fig. 4B, E). In the common crus, the initial ATP secretion per surface area was 8.23 ± 1.88 nM/mm^2, with a ΔATP of 0.05 ± 0.01 nM/mm^2, after the first mechanical stimulation. The ΔATP secretion per surface in the first mechanical stimulation was again significantly higher compared with that of the initial resting, second mechanical stimulation, third mechanical stimulation, and final resting state ($p < 0.001$) (Fig. 4C, F). The ATP secretion was not significant following the second and third stimulation in each tissue ($p > 0.05$), suggesting the ATP reservoir was depleted after the first stimulation.

In the first mechanical stimulation, the ΔATP secretion per surface in the saccule was significantly higher when compared with that in the common crus and utricle with ampulla ($p = 0.001$). However, there was no significant difference in the ΔATP secretion per surface between utricle with ampulla and saccule or utricle with ampulla and common crus ($p > 0.05$) (Fig. 4G).

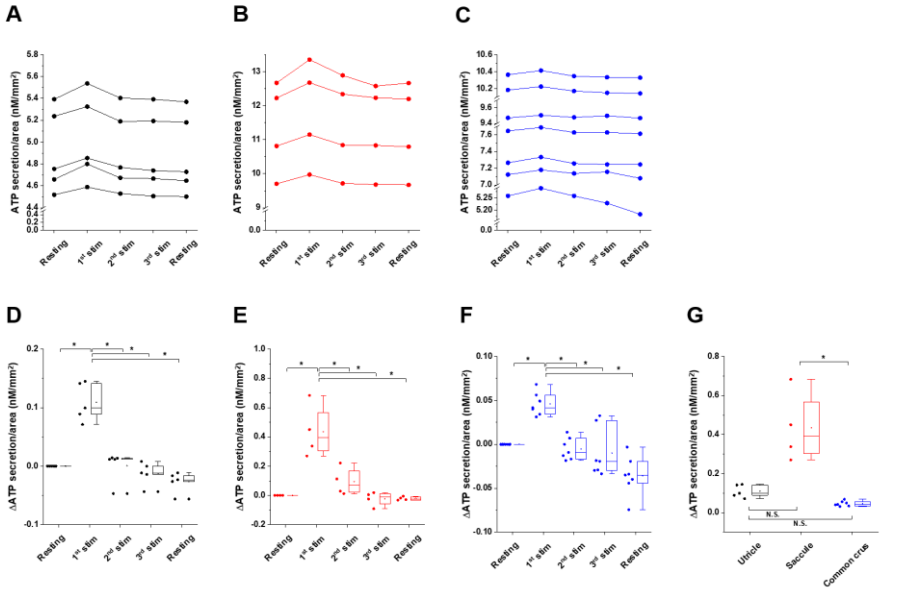

Figure 4. Results of the ATP secretion after mechanical stimulation. (A) Total amount of ATP secretion per surface area in the utricle with ampulla. (B) Total amount of ATP secretion per surface area in the saccule (C) Total amount of ATP secretion per surface area in the common crus. (D) ΔATP secretion per surface area in the utricle with ampulla. (E) ΔATP secretion per surface area in the saccule. (F) ΔATP secretion per surface area in the common crus. (G) ΔATP secretion per surface area of each tissue after first mechanical stimulation. Statistical analysis was performed using one-way ANOVA with Holm–Sidak's post-test or Kruskal–Wallis one-way ANOVA on ranks with Dunn's post-test. *, $p < 0.05$; N.S., not significant.

2. Inhibitor study of the ATP secretion

We investigated the ATP secretion in the mechanical simulation with the

application of CBX (10 nM) or bafilomycin A1 (100 nM) (15-min interval, three times). The initial amount of ATP secretion per surface area after the first mechanical stimulation with CBX was 1.89 ± 0.51 nM/mm^2 in the utricle with ampulla, 3.98 ± 1.44 nM/mm^2 in the saccule, and 3.29 ± 0.60 nM/mm^2 in the common crus (Fig. 6). After CBX application, the ΔATP secretion per surface area was -0.05 ± 0.04 nM/mm^2 in the utricle with ampulla, -0.06 ± 0.22 nM/mm^2 in the saccule, and -0.10 ± 0.26 nM/mm^2 in the common crus, which were all below the detection range. During the second mechanical stimulation, the ΔATP secretion per surface area was not significantly increased in each tissue (Fig. 5). This implicated that ATP secretion was completely blocked by CBX in these three groups

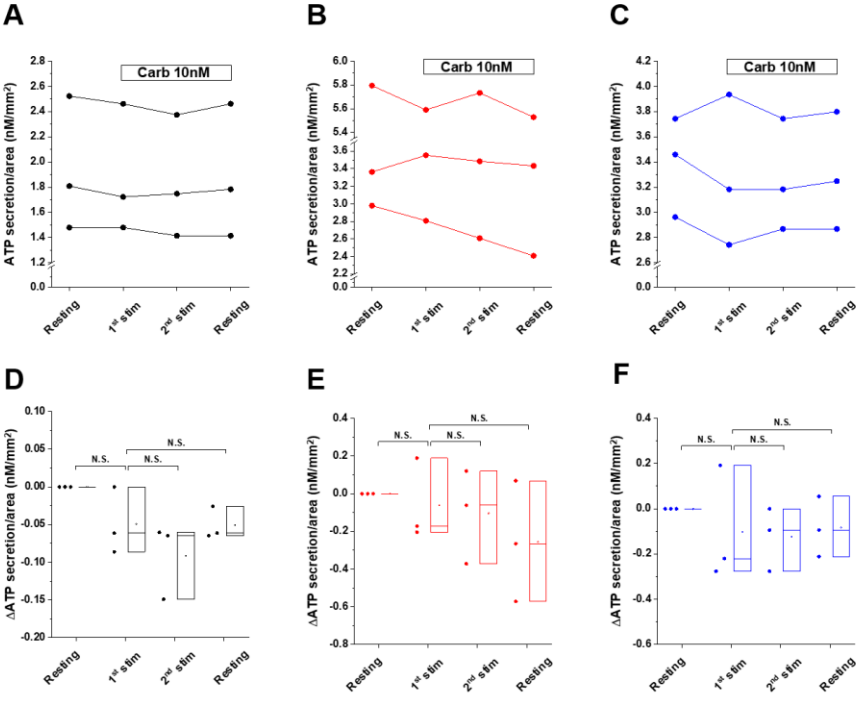

Figure 5. Results of the ATP secretion with the application of carbenoxolone. (A) The amount of ATP secretion per surface area (nM/mm^2) in the utricle with ampulla. (B) The amount of ATP secretion per surface area in the saccule. (C) The amount of ATP secretion per surface area in the common crus. (D) ΔATP secretion per surface area in the utricle with ampulla. (E) ΔATP secretion per surface area in the saccule. (F) ΔATP secretion per surface area in the common crus. Statistical analysis was performed using t-test and one-way ANOVA with Holm–Sidak's post-test or Kruskal–Wallis one-way ANOVA on ranks with Dunn's post-test; N.S., not significant.

We also investigated ATP secretion with the application of bafilomycin A1 (100 nM) in the same manner. The amount of ATP secretion per surface area after the first mechanical stimulation with bafilomycin A1 was -0.04 ± 2.19 nM/mm^2 in the utricle with ampulla, -0.38 ± 5.0 nM/mm^2 in the saccule, and -0.02 ± 0.01 nM/mm^2 in the common crus (Fig. 6A–C). In the utricle with ampulla and saccule, the ΔATP secretion per surface in the first mechanical stimulation was significantly higher than those in the initial resting, second mechanical stimulation, or final resting ($p < 0.001$) (Fig. 6D, E). However, there was no significant difference in ΔATP secretion per surface area before and after bafilomycin A1 application in the common crus ($p > 0.05$) (Fig. 6F).

We investigated the difference in ΔATP secretion per surface area with or without bafilomycin A1 application during mechanical stimulation in each tissue (Fig 6G). Although the ΔATP secretion per surface area seemed to decrease with bafilomycin A1 application, there was no significant difference in

the ΔATP secretion per surface area in saccule ($p > 0.05$). However, the ΔATP secretion per surface area was significantly decreased in utricle with ampulla and common crus ($p = 0.001$).

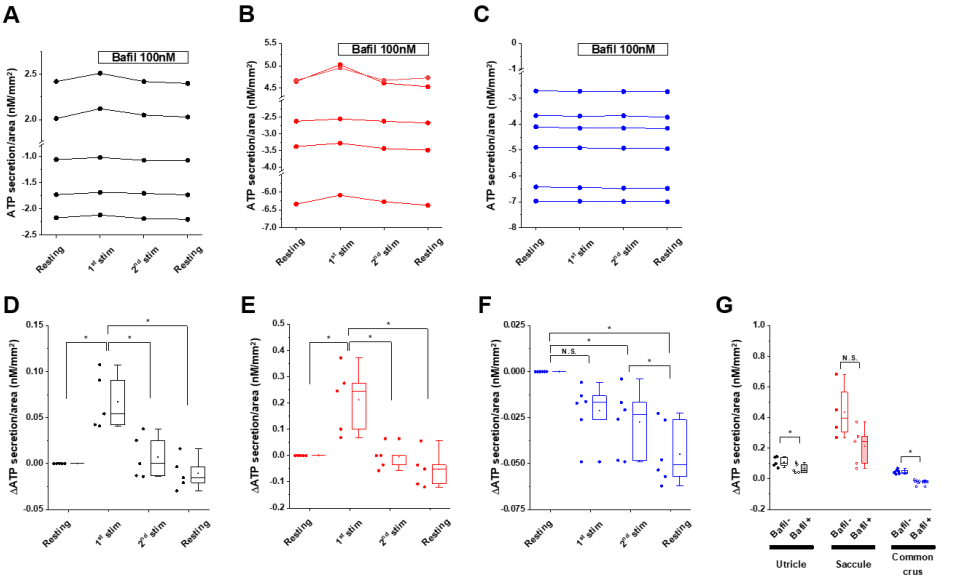

Figure 6. Results of the ATP secretion with bafilomycin A1. (A) The amount of ATP secretion per surface area (nM/mm^2) in the utricle with ampulla. (B) The amount of ATP secretion per surface area in the saccule. (C) The amount of ATP secretion per surface area in the common crus. (D) ΔATP secretion per surface area in the utricle with ampulla. (E) ΔATP secretion per surface area in the saccule. (F) ΔATP secretion per surface area in the common crus. (G) The ΔATP secretion per surface area with or without bafilomycin A1 application in three different tissues. Statistical analysis was performed using t-test and two-way ANOVA with Holm–Sidak's post-test or Kruskal–Wallis one-way ANOVA on ranks with Dunn's post-test for A-F; t-test was performed for G. *, $p < 0.05$; N.S., not significant

3. Quinacrine stain of vestibular organ

Quinacrine staining was performed to identify the accumulation of ATP containing vesicles in the vestibular organ. In the utricle and ampulla, ATP was detected in the dark cell areas in the utricular roof epithelium and utricular macula, the dark cell area around the ampullary crest, and in the ampullary crest itself (Fig 7). In the common crus, ATP was detected only in the area where dark cells were distributed (Fig 8A and B). In the saccule, ATP was detected only in the macular area (Fig. 8C and D). The signal intensity of ATP appeared more intense in the macula of the utricle/saccule and ampullary crest than that in the dark cell areas of the utricular roof epithelium, ampullary, or common crus.

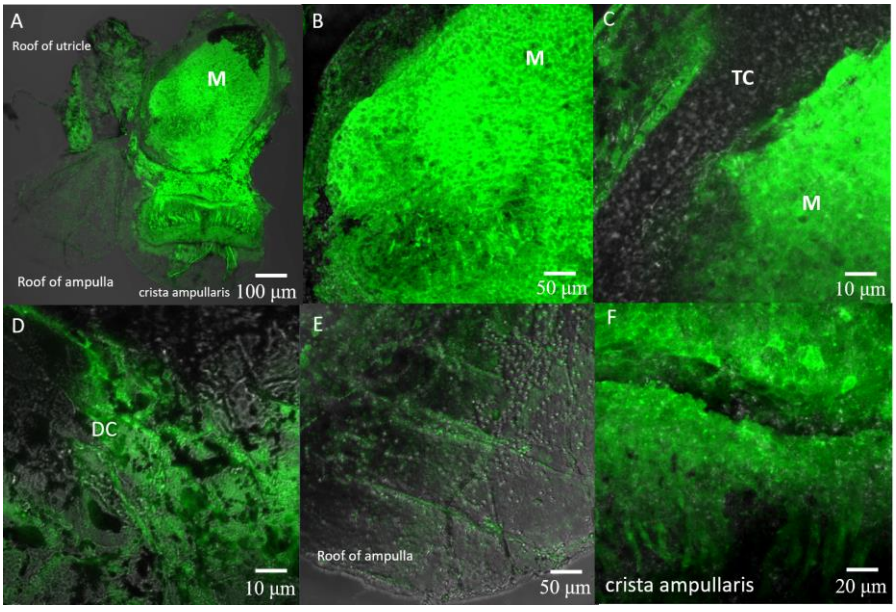

Figure 7. Quinacrine stain of utricle and ampulla. Prepared tissues were stained for quinacrine (5 μM, 3 min) (green). (A) Overall appearance of utricle and ampulla. (B) Utricular macula (M). (C) Utricular macula and transitional

cell area (TC). (D) Utricular roof epithelium with dark cells (DC). (E) Ampulla roof epithelium. (F) Crista ampullariids. Scale bars: 100 μm (A), 50 μm (B, E), 20 μm (F), and 10 μm (C, D).

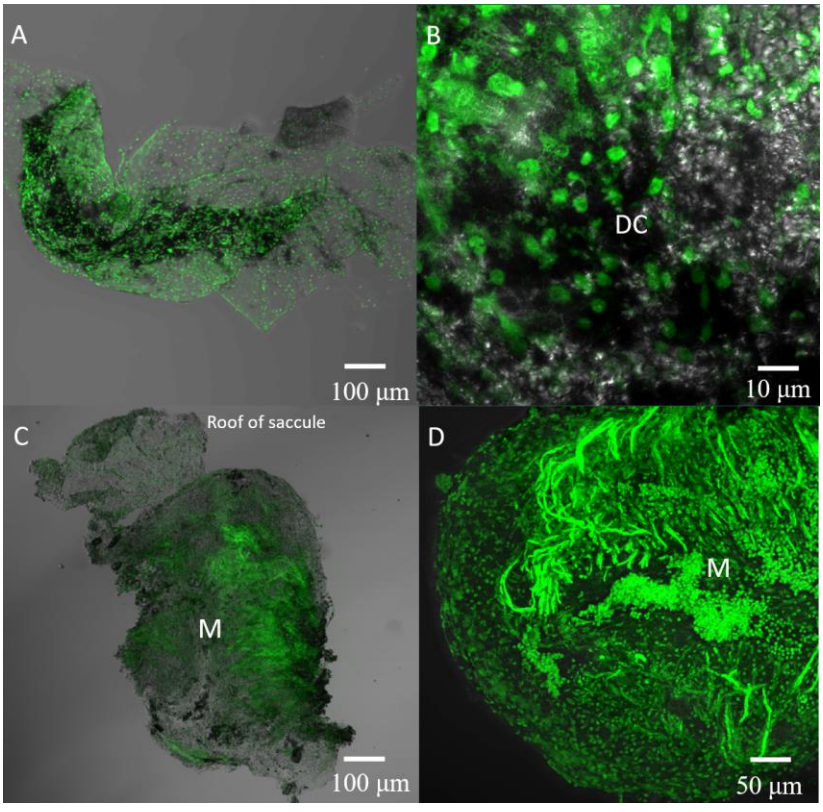

Figure 8. Quinacrine stain of common crus and saccule. Prepared tissues were stained for quinacrine (5 μM, 3 min) (green). (A) Overall appearance of common crus. (B) Dark cell area of common crus. (C) Overall appearance of saccule. (D) Saccular macula (M). Scale bars: 100 μm (A, C), 10 μm (B), and 50 μm (D).

4. Distribution of connexin 26 in vestibular organ

Connexin 26 (CX26) forms a gap junction hemichannel[21] and has been shown to be distributed over the apical membrane of supporting cells of utricular / saccular macula and ampullary crest.[22,23] Since there was no definite evidence of the distribution of CX26 in the vestibular dark cell area, we investigated the distribution of CX26 in mouse vestibular dark cell areas by immunohistochemical staining. CX26 was identified to be consistently distributed in the vestibular dark cell areas in the roof epithelium and common crus (Fig 9). CX26 was distributed over the apical surface of the dark cell areas as shown by 3D z-stack image analysis.

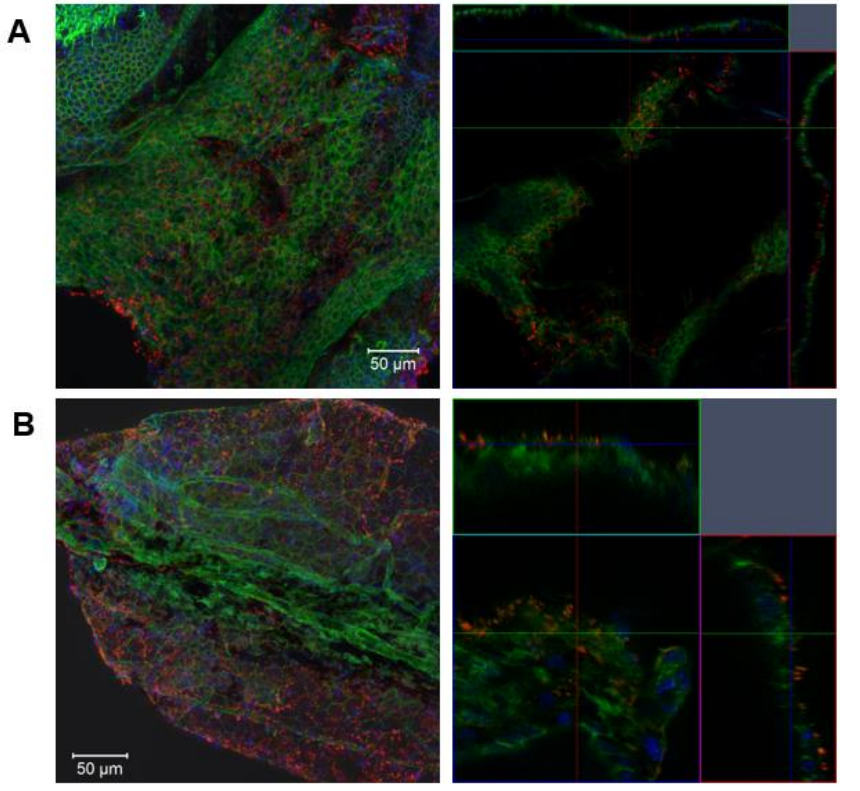

Figure 9. Distribution of connexin 26 (CX26) in vestibular dark cell area of utricle and common crus. (A) Whole mount preparation of utricular dark cell area of roof epithelium. (B) Whole mount preparation of common crus. Prepared tissues and sections were stained for phalloidin (green), CX26 (red) and DAPI (blue). Scale bar: 50 μm.

IV. DISCUSSION

This is the first study to identify ATP secretion in the vestibular organ with direct functional and molecular evidence. The most salient observations of this

study are: 1) The ΔATP secretion per surface area with mechanical stimulation was in the order of saccule > utricle > common crus, 2) ATP secretion with mechanical stimulation was completely blocked by CBX (10 nM) and partially or totally blocked by bafilomycin A1 (100 nM) depending on the subdivision of the vestibular organ, 3) ATP containing vesicles were identified by quinacrine staining in the dark cell area, ampullary crest, utricular macula, and saccular macula, which coincided with the distribution of CX26. Taken together, these observations suggest that ATP was released during mechanical stimulation of the vestibular labyrinth by vestibular dark cells and connexin hemichannels in the vestibular supporting cells and dark cells.

ATP secretion in the cochlea has been proven in animal experiments. Zhao et al. demonstrated that the hemichannels on the cochlear-supporting cells released ATP during mechanical stimulation by applying mechanical stimulation in an *ex vivo* setting;[5] bony shell peeled-off guinea pig cochlea was placed in physiological saline (142 mM NaCl, 5.37 mM KCl, 1.47 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 25 mM dextrose, osmolarity 300 mOsm, pH 7.2) containing micro-glass beads and mechanical stimulation applied by gentle pipetting. Consequently, the cochlear ATP release increased from the $0.95 \pm 0.054 \times 10^{-13}$ to $4.67 \pm 0.71 \times 10^{-13}$ mole with mechanical stimulation, where the ATP concentration in the total volume of the cochlear fluid space was 46.6 ± 2.1 nM. In addition, expression of connexin was detected on both apical and basolateral surfaces of the supporting cells. ATP can be released directly into the perilymph and endolymph from the supporting cells through the hemichannels and can consequently act on P2 receptors on the various sensory and

extra-sensory epithelial cells to modulate signal transduction and inner ear homeostasis.[1,5,24] Although the vestibular system contains functionally similar epithelial cells, evidence for ATP secretion has been lacking. We investigated ATP secretion in the vestibular system using modified measurement and analysis methods. First, instead of using glass beads and gentle pipetting, we used a 3D multi-shaker for the mechanical stimulation. When we used the glass beads and pipetting method, we noticed that the tissue was easily damaged by these techniques and increased the error in the results. Furthermore, consistent mechanical strength could not be provided by manual pipetting. Therefore, we selected a 3D multi-shaker with perilymph-like physiological saline, but without glass beads. This ensured a consistent stimulation that was similar to physiological acceleration stimulus but that avoided tissue damage and reduced the error rate. We found reliable evidence for the existence of ATP secretion in the vestibular organ using these methods. The identified ΔATP secretion amount was 0.11 ± 0.03, 0.44 ± 0.18, and 0.05 ± 0.01 nM/mm^2 for utricle with ampulla, saccule, and common crus, respectively. A significant increase in ATP secretion was observed at the first mechanical stimulation in all tissues and the highest ATP secretion was found in the saccule. The amount of ATP in each tissue was 5.13 ± 0.34, 2.31 ± 0.41, and 2.67 ± 0.47 nM/mm^2 for utricle with ampulla, saccule, and common crus, respectively. The total amount of ATP secretion in our experiment was lower when compared with Zhao et al. This difference may originate from the differences in the stimulation, analysis, tissues, or the animal species. First, glass beads and manual pipetting could apply much stronger mechanical stimulation than rotational stimulation and thereby cause higher

amounts of ATP secretion. Second, we measured the surface area of each tissue before stimulation and quantified ATP secretion per area in every tissue. However, Zhao et al. measured the amount of ATP secretion by mechanical stimulation and calculated the total amount of ATP secretion in the cochlear by using a previously reported volume of guinea pig endolymphatic and peri lymphatic space, and this may have introduced a certain degree of error. Third, mouse and guinea pig may require different amounts of ATP for stimulating purinergic receptors, since the cellular number of the inner ear can be different. Fourth, the vestibular system may not require as much ATP as cochlea, since the physiological response to mechanical stimulation can be different between the two organs.

In the pharmacological study using CBX and bafilomycin A1, CBX completely eliminated the ATP secretion in all the tissues, but bafilomycin exhibited a complete inhibitory effect only on the common crus. ATP secretion was increased even with the application of bafilomycin A1 in utricle with ampulla. This implicates that ATP is mostly released from the connexin hemichannels of the vestibular system. Bafilomycin A1 had little effect in the saccule but had a significant inhibitory effect in the utricle with ampulla and the common crus. The order of the inhibitory effect of bafilomycin A1 was saccule < utricle with ampulla < common crus. From these results, we can assume that the amount of ATP secretion from vestibular dark cell was less than that from connexin hemichannels. This was also supported from the quinacrine staining results that demonstrated a lower signal intensity of ATP in the vestibular dark cell areas than that seen in the macular areas. Therefore, the inhibitory effect of

bafilomycin A1 was significant but smaller in the utricle with ampulla, which contains large number of connexin hemichannels in the macular supporting cells (Fig. 6D and G). In the saccule, there are no dark cells but connexin hemichannels are distributed over macular supporting cells. In the common crus, which contains a high number of dark cells when considering the whole cellular density of the tissue, bafilomycin A1 almost completely blocked the secretion of ATP (Fig. 6C and G). However, CBX also completely inhibited ATP secretion in the common crus and it is difficult to conclude if the ATP was secreted through connexin hemichannels and/or vestibular dark cells. In addition, the limited amount of ATP secretion from the common crus can contribute to these errors.

In our experiment, the saccule secreted a higher amount of ATP per unit surface area when compared with that found in the utricle with ampulla and the common crus. This is an unexpected finding since the utricle and ampulla has a wider area of supporting cells and dark cells, which is a main site for ATP secretion based on the finding of this study. Currently, we cannot explain why there was a high secretion of ATP per unit surface area of saccule. The utricle and the saccule are responsible for sensing linear acceleration, gravitational forces, and tilting of the head, but little is known about the differences and sensitivities according to the purinergic receptors in the different cell types of these tissues. Future studies examining the differences in the role of ATP and purinergic receptors according to the cell types in those organs could elucidate the reason for the difference seen here in the amount of ATP secretion per unit surface area.

The cochlear perilymph and endolymph are known to contain nanomolar

concentrations of ATP that can mediate cochlear and hearing functions. Accordingly, ATP is thought to exist in vestibular organs to modulate balance system. Our experiments demonstrated that vestibular organ could release ATP, providing a potential source of ATP in the vestibular system. Since the vestibular organ is the primary site for managing balance maintenance, the role of the ATP secretion in this system should be of high importance because the dysfunction of this mechanism can cause balance disorders, even in response to ordinary acceleration that occurs in daily life. We believe that the results of this study provide a basis for elucidating normal mechanisms for maintaining balance and understanding pathological mechanisms in balance disorders caused by dysfunction of the ATP secretion in the vestibular system.

V. CONCLUSION

This is the first study to demonstrate that mechanical stimulation induces ATP secretion in the vestibular organs. The main sites of ATP secretion are believed to be the macular gap junction hemichannels and the dark cell areas. The results of this study can justify the role of ATP mediated ion transport via purinergic receptors for various physiological response in the vestibular system during mechanical stimulation.

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ABSTRACT (IN KOREAN)

물리적 자극에 의한 전정기관에서의 ATP 분비의 증거와 위치 규명

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이 현 진

전정기관은 일상 생활에서 가속 자극 중의 균형 유지에 중요하다. 와우의 외림프와 내림프에는 나노몰 농도의 아데노신삼인산(ATP)이 존재하고 있으며, ATP는 다양한 종류의 푸린 수용체를 통해 와우 및 청각 기능의 항상성 유지를 위한 매개체 역할을 한다. ATP는 전정기관에도 존재하며, 내이의 균형 시스템을 와우와 같은 방식으로 조절하는 것으로 여겨진다. 그러나 지금까지 전정기관에서 ATP 분비에 대한 직접적인 증거는 밝혀진 바가 없었다. 따라서 본 연구에서는 전정기관에서 ATP 분비의 증거 및 위치에 대해 알아보고자 한다.

C57BL/6 마우스의 막전정미로를 해부한 뒤, 난형낭, 구형낭, 팽대 및 공통각으로 분리하였다. 각 조직의 단위 면적 당 ATP 분비량을 측정하기 위해, 레이저공초점현미경을 이용하여 각 조직의 표면적을 계산하였다. 전정기관에 물리적 자극을 주기 위해 3D multi-shaker (250 rpm, 15분)를 이용하였다. ATP 분비량은 luciferin-luciferase 분석 키트를 사용하여 Luminometer에 의해 측정되었다. 전정 암흑세포 및 Connexin

hemichannel에 의한 ATP 분비를 억제하기 위해 Bafilomycin A1(100nM) 및 carbenoxolone(CBX)(10nM)을 이용하였다. 전정기관 내의 ATP 분비 위치를 확인하기 위해 Quinacrine 염색을 통해 ATP를 포함하고 있는 소포를 확인하였다. 또한, 면역형광염색을 통해 마우스 암흑세포 영역에 connexin 26 (CX 26) 이 존재하는지 확인하였다.

물리적 자극 시 단위 면적 당 ATP 분비량은 모든 조직에서 유의하게 증가되었다 (난형낭 및 팽대부; 0.11 ± 0.03 nM/mm^2, 구형낭; 0.44 ± 0.18 nM/mm^2, 공통각; 0.05 ± 0.01 nM/mm^2). 구형낭에서의 ATP 분비량은 다른 전정 기관보다 유의하게 높았다. 난형낭 및 팽대부, 구형낭에서의 ATP 분비는 Bafilomycin A1에 의해 부분적으로 억제되는 반면 공통각에서는 완전히 억제되었다. 한편, 모든 조직에서 ATP 분비는 CBX에 의해 완전히 억제되었다. Quinacrine에 의해 염색된 ATP 함유소포는 난형낭의 지붕상피, 난형낭 및 구형낭의 평형반, 팽대부릉정의 암흑세포 영역, 공통각 및 난형낭의 암흑세포 영역에서 나타났다. CX26은 전정기관의 지붕상피 및 공통각의 암흑세포 영역에 일관되게 분포함을 확인하였다.

이 연구는 물리적 자극에 의해 전정기관에서 ATP 분비량이 증가하는 것을 확인한 최초의 연구이다. 전정기관 내 ATP 분비의 주요 부위는 난형낭 및 구형낭의 평형반에 존재하는 gap junction hemichannel과 암흑세포 영역으로 보인다. 본 연구 결과는 전정기관에서 물리적 자극에 의해 ATP 분비가 증가되며, 다양한 푸린 수용체를 통한 ATP 매개 이온 수송이 이루어지는 것을 뒷받침한다.

핵심되는 말: 아데노신삼인산, 전정, 내이, 물리적 자극, 암흑 세포