$P2X_2$ and $P2X_4$ receptor-mediated cation absorption in utricular transitional cells and macula

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Directed by Professor Sung Huhn Kim

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This certifies that the Doctoral Dissertation of Junhui Jeong is approved.

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ABSTRACT

$P2X_2$ and $P2X_4$ receptor-mediated cation absorption in utricular transitional cells and macula

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(Directed by Professor Sung Huhn Kim)

Adenosine 5'-triphosphate (ATP) regulates inner ear function by modulating ion transport through purinergic receptors in inner ear epithelial cells. This study was designed to investigate purinergic receptor-mediated cation transport by mouse utricular macula and the surrounding transitional cells (TCs), where linear acceleration stimuli are sensed. Among ATP, adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP), and uridine 5'-diphosphate (UDP), only ATP (100 μM) induced cation absorption currents in TCs and macula. The current was almost completely inhibited by the application of gadolinium (100 μM). The order of agonist potencies for the cation absorption current was ATP > 3'-*O*-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (bzATP) >> α,β-methyleneadenosine 5' -triphosphate (αβmeATP) in both TCs and macula, and the EC_{50} (concentration that produces a half-maximal effect) values for ATP, bzATP, and αβmeATP were 27.2 μM, 43.9 μM, and 34.5 μM in the TCs and 20.7 μM, 63.4 μM, and 2014.1 μM in the macula, respectively (EC50 values of αβmeATP were not definitively identified due to the low potency of αβmeATP). The ATP-induced current was partially blocked by suramin (100 μM), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) (10 μM), and 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4

-diazepin-2-one (5-BDBD) (5 μM) and was almost completely blocked by PPADS + 5-BDBD in both areas. Immunocytochemistry revealed that $P2X_2$ receptors were distributed in TCs and supporting cells in the macula; however, only $P2X_4$ receptor was not detected in the macula by immunocytochemistry, but only its mRNA expression was detected there. These results indicate that ATP induces cation absorption through $P2X_2$ and $P2X_4$ receptors in utricular TCs and the macula. $P2X_2$ and $P2X_4$-mediated cation transport likely provides a cation shunt under conditions of excessive linear acceleration, thereby protecting hair cells by reducing their cation burden.

Key words : utricle, transitional cell, macula, receptor, cation

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I. INTRODUCTION

The utricle is a part of the vestibular organs of the inner ear and is involved in maintaining balance by detecting linear acceleration. The sensory epithelium of the utricle is called the macula, which is composed of the otolithic membrane, hair cells (HCs), and supporting cells (SCs), and the macula transits into transitional cells (TCs) in the periphery (Figure 1.). Linear acceleration of the head induces movement of the otolithic membrane, which causes the displacement of HC stereocilia. Displacement in the direction of the kinocilium, which is the longest streocilium, modulates the opening of mechano-sensitive non-selective cation channels at the stereocilia, which induces K^+ influx into HCs. This series of movements results in the depolarization of HCs, which causes Ca^{2+} influx through voltage-gated Ca^{2+} channels and finally causes neurotransmitter release at the basolateral surface of HCs to propagate electrical signals to the central nervous system through vestibular nerve fibers.[1] The

generation of electrical signals in HCs to maintain normal balance requires a luminal fluid with a distinct ionic composition (high [K^+] and low [Na^+]), known as the endolymph. The unique ion composition of the endolymph is maintained by the activity of various ion channels in the inner ear epithelial cells and their regulatory materials in the fluid.[2] Adenosine 5'-triphosphate (ATP) is one of the paracrine materials secreted into the endolymphatic space in response to mechanical stimulation to the inner ear, such as by loud noise, and is involved in regulating inner ear ion concentrations through purinergic receptors on the inner ear epithelium.[3] Purinergic receptors are thought to play a role in protecting the inner ear from severe mechanical stress induced by loud noise or excessive acceleration by regulating endolymphatic cation concentrations.[3,4] ATP-stimulated cation absorption through purinergic receptors by inner ear epithelial cells, such as cochlear outer sulcus cells and vestibular ampullary TCs, reduces the K^+ burden on sensory HCs.[5] The utricular macula can exhibit similar purinergic receptor-mediated ion transport, in that both the vestibular ampulla and the utricle have similar cell types, although the role of each organ is different (detection of angular acceleration and linear acceleration for the ampulla and the utricle, respectively). Additionally, purinergic signaling-mediated cation transport is likely to occur more vigorously in the utricle because the surface area of TCs and the sensory epithelium is larger than that of the ampulla. However, nothing has been revealed regarding purinergic receptor-mediated ion transport and receptor types / subunits in the utricular TC.

Moreover, the role of purinergic receptors in the macular region remains unclear.

In this study, we investigated purinergic receptor-mediated ion transport in the utricular TC and macular area using electrophysiological, pharmacological, and molecular biological methods. This study will elucidate the mechanisms of endolymphatic ion concentration regulation as a mechanism by which vestibular organs protect themselves from excessive mechanical stress.

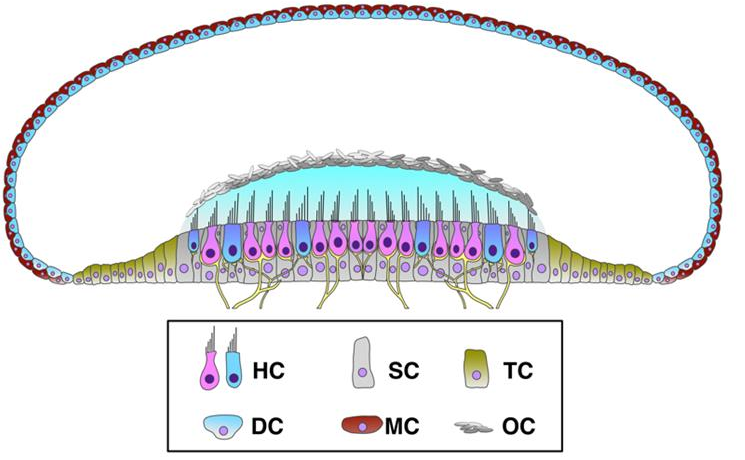

Figure 1. A schematic figure of a cross-sectional view of the utricle. Hair cells (HCs), supporting cells (SCs), and the otolithic membrane, a thin layer of otoconia (OC), comprise the utricular macula. Transitional cells (TCs) are located in the periphery of the macula. The utricular roof epithelium was composed of dark cells (DCs) and melanocytes (MCs).

II. MATERIALS AND METHODS

1. Trans-epithelial current measurement

A. Preparation of the utricle

C57BL/6 mice (6-8 weeks old) were anesthetized by i.p. injection with 30 mg/kg tiletamine-zolazepam (Zoletil®; Virbac, Carros, France) and 10 mg/kg xylazine (Rompun®; Bayer, Leverkusen, Germany) and were sacrificed by decapitation. Temporal bones were dissected, and the complete membranous labyrinth of the vestibule was carefully removed from the temporal bone in perilymph-like physiologic saline (see C. Solution and chemicals). Then, the utricle was excised from the labyrinth, and the utricular roof epithelium was carefully removed. After removal of the roof epithelium, the otolithic membrane was carefully removed from the utricular macula, and the macula was folded with the luminal side facing outward, similar to a previously reported method.[6] The tissue was mounted in a perfusion chamber on an inverted microscope (IX51®; Olympus, Tokyo, Japan) and continuously perfused with perilymph-like physiologic saline at an exchange ratio of 1.1 times/s.

B. Electrophysiological / pharmacological methods

The scanning vibrating electrode technique (SVET) was used to measure the current in the TC and macular areas under short circuit conditions, as reported previously.[6] Each area of the utricular macula was distinguishable via microscopy (×100), and the trans-epithelial current from each cell area was measured by positioning the probe at each area (Figure 2. and 3.). To avoid

interference from the trans-epithelial current from each area, the probe was positioned as far as possible (> 30 μm) from the utricular macula when measuring the current from the TC area, and vice versa. Because the spatial resolution of the SVET system is reported to be 10-30 μm,[7,8] the current interference at these positions was minimal. Current density was monitored using a vibrating platinum-iridium wire microelectrode that was insulated with parlene-C (Micro Electrodes, Gaithersburg, MD, USA) and coated with platinum black on the exposed tip. The electrode tip of the probe was vibrated at two frequencies between 400 and 700 Hz along a horizontal (*x*) and vertical (*z*) axis by piezo-electric bimorph elements (Applicable Electronics, Forestdale, MA, USA) and was positioned 10 ± 2 μm from the apical surface of the epithelium. The *x*-axis was perpendicular to the face of the epithelium. A platinum-black electrode served as a reference in the bath chamber. The signals from the oscillators driving the probe, which were connected to a dual-channel phase-sensitive detector (Applicable Electronics, Forestdale, MA, USA), were digitized (16 bit) at a rate of 0.5 Hz. The electrode was positioned where the current density showed a maximum *x* value and minimum *z* value. Data derived from the *x* direction current density were plotted with Origin software, version 8.0. (OriginLab Software, Northampton, MA, USA).

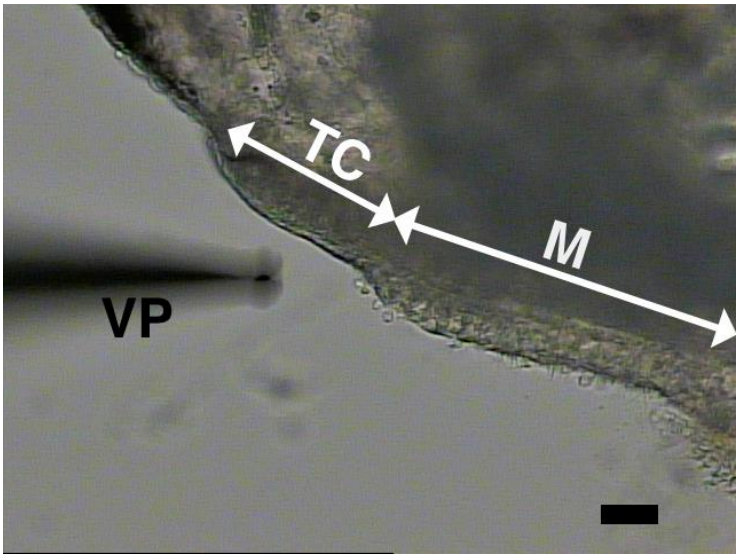

Figure 2. A representative figure of trans-epithelial current measurements from the utricular macula using the scanning vibrating electrode technique. The trans-epithelial current under short circuit conditions was measured in the transitional cell (TC) area or mucular (M) area, which was easily identified by the presence of stereocilia. VP: vibrating probe. Scale bar: 50 μm.

C. Solution and chemicals

For electrophysiological experiments, a perilymph-like physiological saline solution [150 mM NaCl, 3.6 mM KCl, 1 mM MgCl2, 0.7 mM $CaCl_2$, 5 mM glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4)] was used for perfusion. ATP (A9187; Sigma, St. Louis, MO, USA), α,β-methyleneadenosine 5'-triphosphate (αβmeATP) (M6517; Sigma, St. Louis, MO, USA), 3'-*O*-(4-benzoyl-benzoyl)adenosine 5'-triphosphate

(BzATP) (B6396; Sigma, St. Louis, MO, USA), uridine 5'-triphosphate (UTP) (U6875; Sigma, St. Louis, MO, USA), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) (P178; Sigma, St. Louis, MO, USA), suramin (S2671; Sigma, St. Louis, MO, USA), and gadolinium chloride ($GdCl_3$) (G7532; Sigma, St. Louis, MO, USA) were directly dissolved in perilymph-like physiological saline just before use. Adenosine 5'-diphosphate (ADP) (A2754; Sigma, St. Louis, MO, USA) and uridine 5'-diphosphate (UDP) (U4125; Sigma, St. Louis, MO, USA) were pre-incubated for 1.5-2 hr at room temperature with hexokinase (1 U/ml) (H4502; Sigma, St. Louis, MO, USA) and glucose (5 mM) to eliminate the possibility of minor ATP and UTP contamination. All purinergic agonists were applied to the bath briefly (7-10 sec) to avoid receptor desensitization and applied once more after the application of all chemicals to confirm receptor activity. Bumetanide (10 μM) (B3023; Sigma, St. Louis, MO, USA), a Na^+-K^+-$2Cl^-$ cotransporter inhibitor, was continuously perfused during the experiment in the TC area to prevent any possible current density interference by the K^+ secretion current from the adjacent dark cell area.

D. Data analysis

Results are presented as the mean ± standard error from n observations. Purine and pyrimidine-induced current was calculated by subtracting the baseline current from the peak point of the purine and pyrimidine-induced current. Baseline current was calculated by averaging the current density from 1 to 1.25

min, when the probe was located at the apical side of each TC and HC area. The significance of the current changes between the two points (baseline and peak point of the induced-current) was calculated using Student's t-test. The effects of purinergic receptor blockers were calculated by subtracting the peak value after ATP application during the perfusion of purinergic receptor blockers from the peak point after ATP application without the application of purinergic receptor blockers. The differences in the effects of the purinergic receptor blockers were calculated using one-way analysis of variance (ANOVA) with Holm-Sidak's post-test. $p<0.05$ was considered significant. The concentration dependence of the purine agonists was analyzed using the Hill equation; $I=I_{max}[C^{h}/(EC_{50} + C^{h})]$, where I_{max} is current in the presence of saturating concentrations of the agonist, C is the concentration of the agonist, h is the Hill coefficient, and EC_{50} is the concentration that produces a half-maximal effect.

2. Immunocytochemistry

A. Fixation of vestibular labyrinths for cryosections

Deeply anesthetized mice were sacrificed by decapitation. The vestibular labyrinth was isolated by microdissection in Cl^--free solution and transferred to fixative. The Cl^--free solution contained 150 mM Na-gluconate, 1.6 mM K_2HPO_4, 0.4 mM KH_2PO_4, 4 mM Ca-gluconate$_2$, 1 mM $MgSO_4$ and 5 mM glucose, pH 7.4. The fixative consisted of 4% formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffered saline (PBS),

which contained 137 mM NaCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4. The isolated vestibular labyrinths were fixed for 1-2 hr at room temperature.

B. Immunocytochemistry of the cryosections

Fixed tissues were processed through a sucrose gradient, infiltrated with polyethylene glycol, and then sectioned (6 μm) (CM3050S®; Leica, Nussloch, Germany). The cryosections were blocked with 5% BSA in PBS-TX (PBS with 0.15% Triton X-100 and 5% bovine serum albumin). The slides were incubated overnight at 4℃ with primary antibody in 2.5% BSA PBS-TX. The following primary antibodies were used: unconjugated rabbit anti-$P2X_2$ (1:200, APR-003; Alomone Labs, Jerusalem, Israel) and Atto-594 conjugated rabbit anti-$P2X_2$ (1:200, APR-003-AR; Alomone Labs, Jerusalem, Israel), which were raised against (C)SQQDSTSTDPKGLAQL (amino acids 470-485 of the mouse $P2X_2$ receptor, GenBank: Q8K3P1), unconjugated rabbit anti-$P2X_4$ (1:200, APR-002; Alomone Labs, Jerusalem, Israel) that was raised against the intracellular epitope (C)KKYKYVEDYEQGLSGEMNQ (amino acids 348-366 of the mouse $P2X_4$ receptor, GenBank: Q9JJX6.1), unconjugated rabbit anti-$P2X_4$ (1:200, APR-024; Alomone Labs, Jerusalem, Israel) that was raised against the extracellular epitope (C)RDLAGKEQRTLTK (amino acids 301-313 of the mouse $P2X_4$ receptor, GenBank: Q9JJX6.1), and unconjugated goat anti-$P2X_4$ (1:100, ab134559; Abcam, Cambridge, MA, USA) that was raised against

(C)ETDSVVSSVTTKAK (amino acids 56-69 of the mouse $P2X_4$ receptor, GenBank: Q9JJX6.1). Slides were washed with PBS-TX and incubated for 1 h at room temperature with phalloidin 488 (1:40; Invitrogen, Carlsbad, CA, USA), 4',6-diamidino-2-phenylindole (DAPI) (1:1,000; Invitrogen, Carlsbad, CA, USA), and, if appropriate, secondary antibody (1:1,000, goat anti-rabbit Alexa 594; Invitrogen, Carlsbad, CA, USA). After staining, the slides were washed and cover-slipped with mounting medium (FluorSave, Cat#: 345789; EMD-Millipore, Darmstadt, Germany).

C. Whole-mount fixation and immunocytochemistry

Vestibular labyrinths were isolated by microdissection in Cl^--free solution. Utricular maculae were isolated, and otolithic membranes were manually removed using fine glass needles. Isolated utricular maculae that were free of otoconia material were fixed for 1-2 hr at room temperature. The fixed tissues were washed in PBS-TX, blocked for 1 h with 5% BSA in PBS-TX, and incubated overnight at 4℃ with primary antibodies in 2.5% BSA PBS-TX. The tissues were washed with PBS-TX and incubated for 1 hr at room temperature with phalloidin 488 (1:40), DAPI (1:1,000), and where appropriate, with secondary antibody (1:1,000, goat anti-rabbit Alexa 594). After staining, the utricular maculae were washed and cover-slipped with mounting medium (FluorSave) on slides that were prepared with nail-polish to act as a spacer to avoid squeezing of the tissue.

D. Confocal microscopy

Immunocytochemistry of cryosections and whole mounts was viewed by confocal microscopy (LSM 880® ; Carl Zeiss, Jena, Germany).

3. Reverse transcription polymerase chain reaction (RT-PCR)

$P2X_4$ receptor transcripts were detected using RT-PCR. Utricles were carefully dissected from the mouse temporal bone, and the roof epithelium was carefully removed as described above. After homogenization of the harvested utricles, total ribonucleic acid (RNA) was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The quantity and quality of the isolated RNA were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and by analyzing the 18S and 28S ribosomal RNA (rRNA) bands after electrophoresis. Complementary deoxyribonucleic acid (cDNA) was synthesized from 3 μg of total RNA using random hexamer primers (Perkin Elmer Life Sciences, Boston, MA, USA; and Roche Applied Science, Mannheim, Germany), Avian myeloblastosis virus (AMV) reverse transcriptase (Perkin Elmer Life Sciences, Boston, MA, USA), and ribonuclease (RNase) inhibitor (Perkin Elmer Life Sciences, Boston, MA, USA). Reverse transcription was performed for 10 min at room temperature, 30 min at 50℃, and 15 min at 95℃. The $P2X_4$ receptor messenger RNA (mRNA) was amplified using gene-specific primers (Table 1.).

The PCR conditions included 30 cycles of denaturation at 94℃ for 30 s, annealing at 59℃ for 30 s, and polymerization at 72℃ for 30 s. The PCR products were run on a 1.5% agarose gel and visualized with ethidium bromide under a transilluminator. The PCR products were purified with a PCR purification kit (Qiagen, Valencia, CA, USA) and the purified PCR products were sequenced to verify the identity of the RT-PCR product.

Table 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR)

Gene	GenBank accession number		Primer (5' → 3')	Amplicon size (base pair)
18S RNA	BK000964	Forward	GAGGTTCGAAGACGATCAGA	315
		Reverse	TCGCTCCACCAACTAAGAAC	
$P2X_4$	NM_001310720	Forward	AGTGGGACTGCAACCTTGAC	219
		Reverse	CAAACTTGCCAGCCTTTCCAA	
		Forward	AAGTGGGACTGCAACCTTGA	222
		Reverse	GTCAAACTTGCCAGCCTTTCC	

RNA: ribonucleic acid.

III. RESULTS

1. Cation absorption in the utricular TCs and macula

K^+ secretion currents were detected when the probe was moved close to the dark cell area of the roof epithelium, and the current decreased as the probe was moved from the dark cell area to the TC area (Figure 3.). The current vector changed to cation absorption when the probe was located in the TC area, and the cation absorption current continued until the probe was moved to the macula (Figure 3.). The mean density of the current was -6.4 ± 1.0 μA/cm^2 in the TC area (n=34) and -6.9 ± 1.0 μA/cm^2 in the macula (n=47).

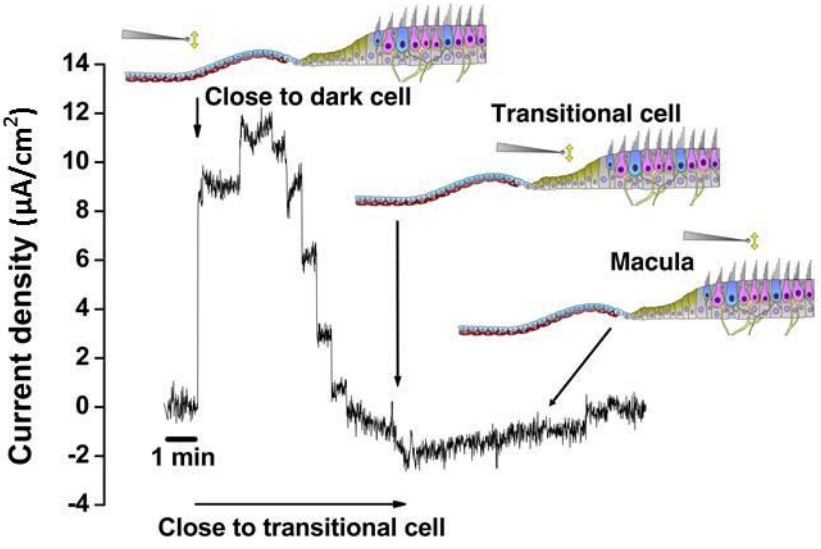

Figure 3. Trans-epithelial current changes while a measuring electrode was moved from the dark cell area (roof epithelium) to the utricular macula (10μm/30s). Cation secretion current from the dark cell area changed into cation absorption current in the transitional cell and macular areas.

2. Effect of purines and pyrimidines on cation absorption in utricular TCs and macula

When purines and pyrimidine such as ATP (100 μM), ADP (100 μM), UTP (100 μM), and UDP (100 μM) were perfused, the cation absorption currents were only significantly induced after ATP application in both the TC area and the macula [amount of current change: -161.7 ± 50.6 μA/cm^2 in the TC area (n=5) and -65.2 ± 22.1 μA/cm^2 in the macula (n=5), p<0.001, Figure 4.]. The effects of the other agonists, which mainly act on mouse P2Y receptors, on current density were insignificant (p>0.05, Figure 4.). This result implies that ATP modulated cation absorption mainly through P2X receptors.

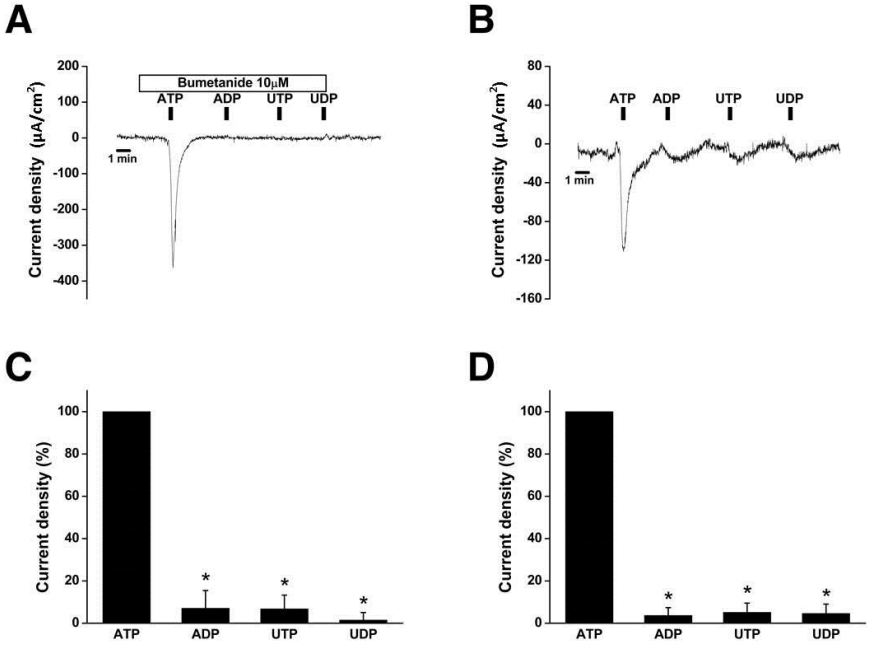

Figure 4. Effects of purines and pyrimidines on trans-epithelial currents from

transitional cells and the macula. A, B. Representative figures of the current change after the application of purines and pyrimidines in transitional cells and the macula, respectively. C, D. Summary of the current density change ratios after purine and pyrimidine application on transitional cells and the macula, respectively (n=5 for each experiment). $^{*}p<0.05$. ATP: Adenosine 5'-triphosphate, ADP: Adenosine 5'-diphosphate, UTP: uridine 5'-triphosphate, UDP: uridine 5'-diphosphate.

3. ATP-induced cation absorption through non-selective cation channels

We investigated if ATP-induced cation absorption occurred through non-selective cation channels formed by P2X receptors, as shown previously in ampullary TCs.[5] Application of Gd (100 μM) significantly decreased the ATP-induced cation absorption current in both the TC and macula areas [ATP-induced current decreased to 21.2 ± 5.3% and 21.4 ± 9.4% of that in the TC area (n=6) and macula (n=5), respectively. $p<0.001$, Figure 5.]. This result indicates that ATP increased cation-absorption mainly through non-selective cation channels (e.g., P2X receptors) in TCs and in other epithelial cells in the macula.

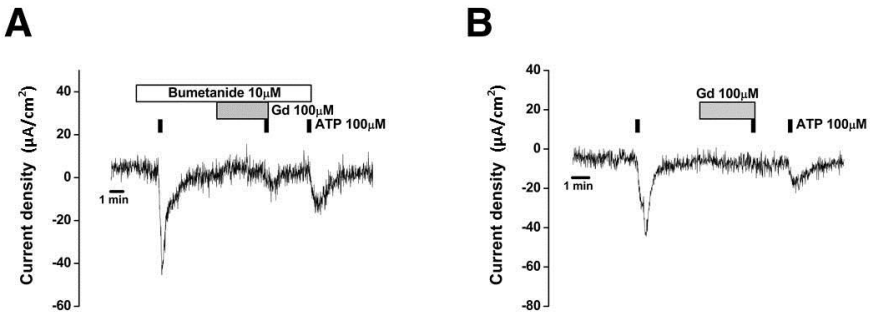

Figure 5. Representative figure of the gadolinium effect on ATP-induced cation absorption in transitional cells (A) and macula (B). Gd: gadolinium, ATP: Adenosine 5'-triphosphate.

4. Functional evidence of $P2X_2$ and $P2X_4$ receptor-mediated cation absorption in utricular TCs and the macula

To investigate P2X receptor subunits in utricular TCs and in other epithelial cells in the macula, agonist potencies using ATP analogues, such as ATP, bzATP, and αβmeATP, were compared. Dose-response experiments revealed that the order of agonist potencies for cation absorption current was ATP > bzATP >> αβmeATP in both the TC area and macula (Figure 6.). The EC_{50} values calculated from the Hill equation for ATP, bzATP, and αβmeATP were 27.2 μM, 43.9 μM, and 34.5 μM (not definitively identified due to the low potency of αβmeATP) in the TC area and 20.7 μM, 63.4 μM, and 2014.1 μM (not definitively identified due to the low potency of αβmeATP) in the macula (n=4 for each experiment), respectively. The order of the agonist potencies and EC_{50}

values were consistent with the reported characteristics of $P2X_2$ and $P2X_4$ receptors (reported EC_{50} values of ATP, bzATP, and αβmeATP are 2-8, 6-30, and >100 μM for $P2X_2$ and 1-10, 3, and 4-300 μM for $P2X_4$ receptor).[9]

Then, we applied P2X receptor blockers to investigate which subunit mediated ATP-induced cation absorption. We first used suramin (100 μM) and PPADS (10 μM) because these concentrations have been shown to completely block $P2X_2$ receptor function while minimally affecting $P2X_4$ receptor activity.[9] The application of suramin and PPADS partially decreased ATP-induced cation absorption both in the TC area and the macula [suramin decreased the ATP-induced current to 47.0 ± 8.8% and 33.1 ± 4.6% of that in the TC area (n=5) and macula (n=5), respectively; PPADS decreased the ATP-induced current to 46.1 ± 4.4% and 41.8 ± 3.8% of that in the TC area (n=5) and macula (n=5), respectively; $p<0.05$, Figure 7A, B, and E.; Figure 8A, B, and E.]. ATP-induced cation absorption current was also partially decreased both in the TC area and macula when 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro [3,2-e]-1,4-diazepin-2-one (5-BDBD) (5 μM), a specific $P2X_4$ receptor blocker, was applied [ATP-induced current decreased to 46.8 ± 5.8% and 41.6 ± 8.8% of that in the TC area (n=5) and macula (n=5), respectively, $p<0.05$, Figure 7C and E.; Figure 8C and E.]. Combined application of PPADS and 5-BDBD almost completely inhibited ATP-induced cation absorption in both the TC area and macula [current decreased to 6.0 ± 4.9% and 9.9 ± 7.8% of that in the TC area (n=5) and macula (n=5), respectively, $p<0.05$, Figure 7D and E.; Figure 8D and

E.]. These results imply that both the $P2X_2$ and $P2X_4$ receptors modulated ATP-induced cation absorption in TCs and the macula.

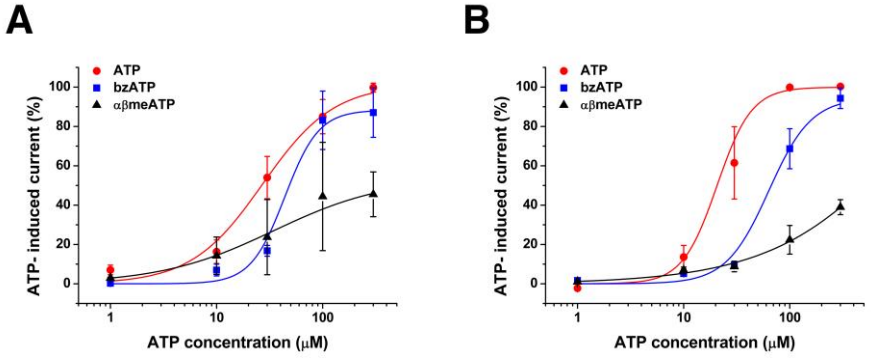

Figure 6. Concentration-response curves for increased current density by ATP analogues in transitional cell (A) and macula (B) areas. Curves are best fits to the Hill equation (n=4 for each experiment). ATP: Adenosine 5'-triphosphate, bzATP: 3'-*O*-(4-benzoyl-benzoyl) adenosine 5'-triphosphate, αβmeATP: α,β-methyleneadenosine 5' –triphosphate.

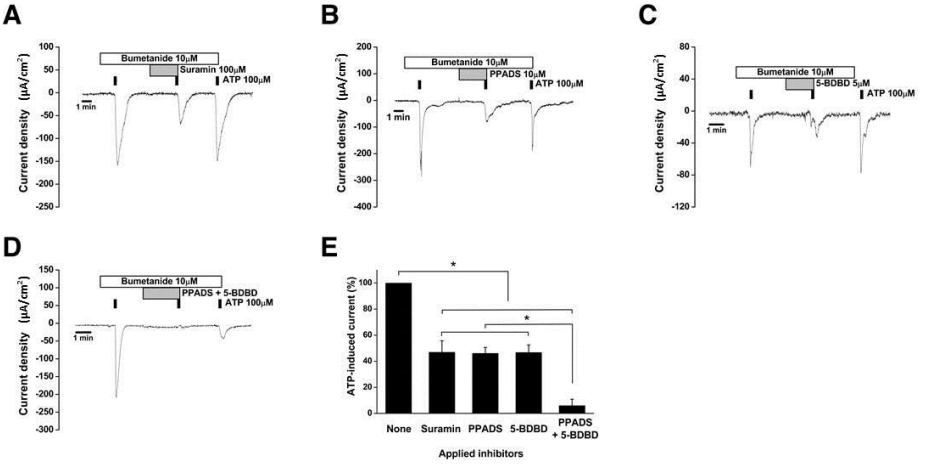

Figure 7. Inhibitory effect of P2X receptor blockers on ATP-induced current

density in the transitional cell area. A-D. Representative figures for the effects of suramin (100 μM), PPADS (10 μM), 5-BDBD (5 μM), and PPADS + 5-BDBD on ATP- induced current density. E. Summary of the inhibitory effects of the blockers on ATP-induced current density (n=5). $^{*}p<0.05$. ATP: Adenosine 5'-triphosphate, PPADS: pyridoxal phosphate-6-azo(benzene- 2,4-disulfonic acid), 5-BDBD: 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro [3,2-e]-1,4-diazepin-2-one.

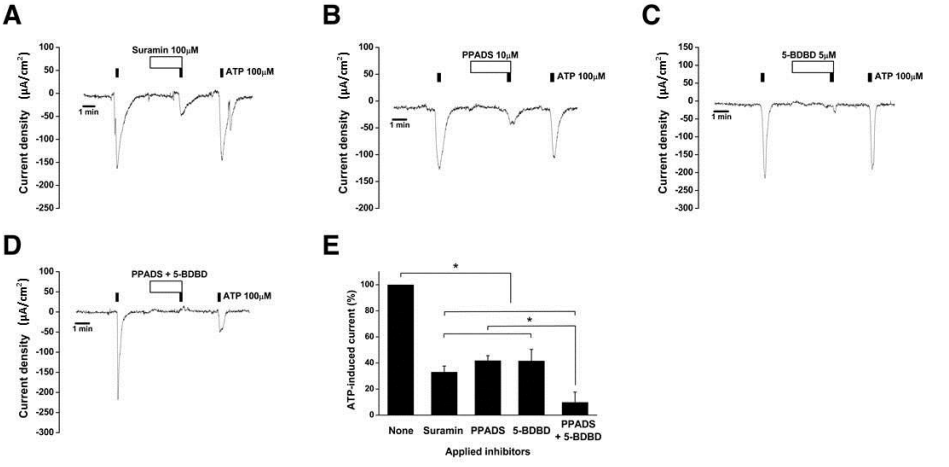

Figure 8. Inhibitory effect of P2X receptor blockers on ATP-induced current density in the macula. A-D. Representative figures for the effects of suramin (100 μM), PPADS (10 μM), 5-BDBD (5 μM), and PPADS + 5-BDBD on ATP-induced current density. E. Summary of the inhibitory effects of the blockers on ATP-induced current density (n=5). $^{*}p<0.05$. ATP: Adenosine 5'-triphosphate, PPADS: pyridoxal phosphate-6-azo(benzene- 2,4-disulfonic

acid), 5-BDBD: 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro [3,2-e]-1,4-diazepin-2-one.

5. Cell-specific distribution of $P2X_2$ and $P2X_4$ receptors in utricular TCs and the macula

Consistent with previous results,[5] $P2X_2$ receptors were identified in TCs of the vestibular ampulla (Figure 9A and C.). In the utricle, receptor expression was detected in TCs and SCs in the macula, but not in HCs in the macula (Figure 9B, D-J.). This finding suggested that the ATP-induced cation absorption current is mediated by $P2X_2$ receptors in utricular TCs and SCs. For $P2X_4$ receptors, despite using several antibodies, we were unable to identify specific $P2X_4$ receptor expression in the ampulla and utricle. However, further examination using RT-PCR revealed the presence of $P2X_4$ mRNA expression in the utricle (Figure 10.), although we were unable to identify the definite location of $P2X_4$ receptors in the utricle. Therefore, whether the $P2X_4$-mediated current originated from SCs or HCs in the macula remains unclear, although the current clearly originated from TCs, because no other cell types are present in the TC area in utricle.

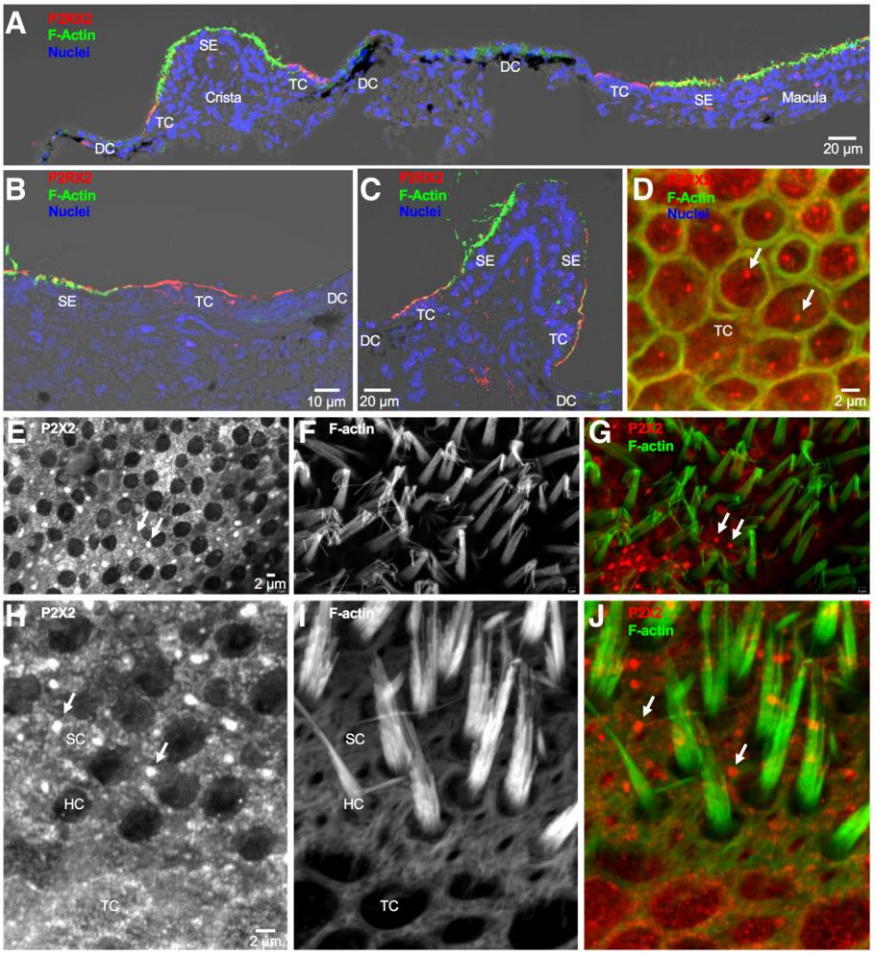

Figure 9. Immunocytochemistry of $P2X_2$ in the vestibular labyrinth. Data shown were obtained using a fluorescent-dye conjugated anti-$P2X_2$ antibody. Similar observations were made using an unconjugated anti-$P2X_2$ antibody. A-C. Cryosections of the utricular macula and the crista of the anterior semicircular canal. $P2X_2$ (red) expression was detected in the apical membrane of transitional cells (TCs) and in the apical membranes of cells in the sensory epithelium (SE). Transitional cells are situated between the sensory epithelium

and vestibular dark cells (DC). The sensory epithelium was marked by heavy filamentous actin (F-actin) staining (green). Vestibular dark cells were identified as epithelial cells overlaying pigmented cells in the underlying connective tissue. D. A whole-mount of the utricular macula. $P2X_2$ expression (red) was observed throughout the apical membranes of TCs. In many cells, $P2X_2$ expression was concentrated in hot spots (arrows). Individual TCs were outlined with F-actin expression (green) near tight junctions. E-J. Whole-mounts of the utricular macula. $P2X_2$ expression (white or red) in the sensory epithelium was found in the apical membranes of supporting cells (SCs) and TCs but not in sensory hair cells (HCs). Sensory HCs were identified by F-actin staining (white or green) of hair bundles. $P2X_2$ expression in the apical membranes of many SCs appeared to be concentrated in hot spots (arrows).

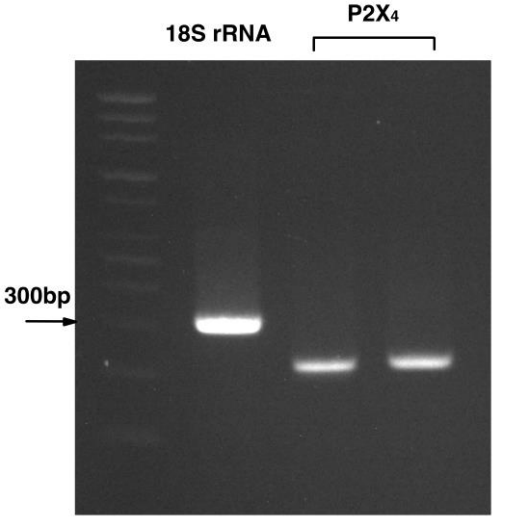

Figure 10. $P2X_4$ receptor mRNA expression in the utricle (RT-PCR). Transcripts were detected using two different primers for $P2X_4$ receptor. mRNA: messenger ribonucleic acid, rRNA: ribosomal ribonucleic acid, bp: base pair.

IV. DISCUSSION

ATP is reportedly secreted in response to excessive noise stimulation in the cochlea, where sound stimulation is transformed into electrical signals to propagate the transduction of the stimulus to the central nervous system.[10] The main sites for ATP secretion were suggested to be strial marginal cells, supporting cells, and the organ of Corti,[11,12] and secreted ATP modulates signal transduction by HCs via $P2X_2$ and $P2Y_2$ receptors by regulating cation (mainly K^+) secretion and absorption in hair cells and neighboring epithelial cells.[3,12] Similarly, the modulation of cation secretion and absorption by ATP via $P2X_2$ and $P2Y_2$ receptors was observed in the vestibular system of gerbils through functional studies. ATP induces cation absorption in ampullary TCs via $P2X_2$ receptors and inhibits cation secretion in utricular dark cells via $P2Y_2$ receptors, which ultimately reduces the cation burden on vestibular HCs.[5] Although ATP secretion in the vestibular system has not been definitively identified, ATP is expected to be secreted by vestibular dark cells and supporting cells because of the structural and functional similarity between strial marginal cells/cochlear supporting cells and vestibular dark cells/vestibular supporting cells.[13] ATP is likely to be involved in the modulation of cation transport in vestibular epithelial cells following excessive mechanical stimulation, such as abrupt

acceleration, as occurs in cochlear epithelial cells. Although purinergic receptor-mediated cation transport was reported in ampullary TCs and utricular dark cells,[3] there are more studies about purinergic receptor-mediated ion transport in the cochlea than in the vestibular system. However, the vestibular organ is the primary site for managing balance maintenance; thus, the role of these receptors in this system should be very important because the dysfunction of these receptors can cause balance disorders, even in response to ordinary acceleration that occurs in daily life.

In the present study, we identified ATP-induced cation absorption in the TCs and macula of the mouse utricle. A unique finding of this study is the co-involvement of $P2X_4$ receptors in addition to $P2X_2$ receptors in ATP-induced cation absorption in utricular TCs, which differs from the finding in ampullary TCs, where only $P2X_2$ receptor involvement in current was identified. Furthermore, this study is the first to functionally identify ATP-induced cation absorption mediated by $P2X_2$ and $P2X_4$ receptors in the utricular macula. $P2X_2$ receptors were distributed in macular supporting cells, although the specific cell types in the macular where the $P2X_4$ receptors were distributed remains inconclusive based on our immunocytochemistry results. The precise localization of the $P2X_4$ receptors in macula epithelial cells should be determined in the future using patch clamp techniques for different cell types or immunostaining using appropriate antibodies. Indeed, purinergic receptor expression on utricular supporting cells was suggested in two functional

studies;[14,15] however, these studies focused on the role of intracellular otopetrin 1 in regulating Ca^{2+} transport through P_2X and P_2Y receptors for otoconia formation and did not further evaluate the roles of specific purinergic receptor subunits. These supporting cells likely expressed $P2X_2$ or both $P2X_2$ and $P2X_4$ based on the results of our study.

The electrophysiological and pharmacological findings in the present study identified non-selective cation absorption via $P2X_2$ and $P2X_4$ receptors in utricular TCs and macula. The evidence for the involvement of these receptors can be summarized as follows: First, the cation absorption current was induced only by ATP, but not by ADP, UTP, and UDP. Second, the ATP-induced cation absorption current was fully inhibited by Gd. Third, the EC_{50} values for the ATP derivatives were close to those of the $P2X_2$ and $P2X_4$ receptors. In fact, the EC_{50} values were closer to the $P2X_2$ receptor, but the presence of $P2X_4$ receptors could not be completely excluded if $P2X_4$ receptors co-existed with $P2X_2$ receptors, because the responses mediated by both receptors could be mixed. Therefore, we attempted to use different P2X receptor inhibitors (100 μM suramin and 10 μM PPADS for P2X2, and 5 μM 5-BDBD for $P2X_4$), and the pharmacological data obtained using these reagents revealed the involvement of both $P2X_2$ and $P2X_4$ receptors in ATP-induced cation absorption. Although most findings indicated the involvement of $P2X_2$ and $P2X_4$ receptors in cation transport in these areas, the existence of P2Y receptors in supporting cells and/or HCs cannot be completely excluded because minimal current changes

with a gentle slope, which can be observed in P2Y receptor-mediated cation absorption induced by ADP, UTP, and UDP, were identified in some cases in the HC area (Figure 4B., n=2). However, the current was not always observed in our experiments, and the current changes were tiny; therefore, the contribution of P2Y receptors to ATP-induced cation absorption is likely minimal.

The $P2X_2$ and $P2X_4$ receptors present in TCs and supporting cells likely provide a cation shunt for the regulation of inward current in HCs by ATP stimulation under conditions of excessive linear acceleration, although the localization of $P2X_4$ receptors in supporting cells remains inconclusive. This mechanism may both protect HCs and regulate HC sensitivity, which is very important in the maintenance of the vestibular system, especially in situations featuring excessive, abrupt acceleration stimuli, which can occur during driving, whiplash injuries during traffic accidents, aviation, space flight, and so forth. If this fine regulation by purinergic receptors is disrupted, vestibular HCs can be damaged, which ultimately leads to dizziness and balance disorders. Indeed, targeted disruption of the $P2X_2$ receptor gene in mice caused progressive hearing loss and increased susceptibility to noise stimulation without recovery.[16,17] Furthermore, $P2X_2$ receptor gene mutations in human are also reported to cause genetic progressive sensorineural hearing loss with an autosomal dominant inheritance pattern.[17] This observation implies that the $P2X_2$ receptor plays an important role in cochlear HC protection. Similarly, disruption of the $P2X_2$ and/or $P2X_4$ receptor gene may cause progressive or

mechanical stress (acceleration stimulus)-induced vestibular dysfunction by causing loss of their protective mechanisms, because those receptors are distributed in vestibular TCs and the macula, which are adjacent to sensory HCs. However, there is no study investigating vestibular function in the mouse model and patients with the $P2X_2$ receptor gene mutation. The future study about vestibular function in the animal model and patients will provide a clue in identifying the role of $P2X_2$ and $P2X_4$ receptors in vestibular function maintenance.

$P2X_4$ receptors may also be distributed in utricular HCs. Although several reports have demonstrated ATP-induced inward current and the presence of purinergic receptors in cochlear inner and outer HCs in different kinds of animals ($P2X_2$, $P2X_3$, and $P2X_7$ in inner HCs and $P2X_1$, $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ in outer HCs),[4,12,18] no studies have evaluated vestibular HCs. In cochlear HCs, purinergic receptors modulate sound transduction and neurotransmission by regulating inward currents and intracellular Ca^{2+} concentrations.[4] Additionally, purinergic receptors are reportedly involved in regulating outer HCs motility, which is important in the amplification of basal membrane movement.[12,18] Because ATP is expected to be secreted in the vestibular system in response to acceleration stimulation, the purinergic receptor-mediated inward current in utricular HCs likely functions to modulate their sensitivity according to the severity of the linear acceleration stimulus.

Consequently, our study revealed that $P2X_2$ and $P2X_4$ receptor-mediated

cation absorption in utricular TCs and macula may serve as a protective or modulatory mechanism for the vestibular system. Although the phenomenon and role of purinergic signaling-induced cation transport in the vestibular system have yet to garner as much interest as those in the cochlea, they nevertheless likely play an important role in the maintenance of balance, as they do in the cochlea. Future further studies of $P2X_2$ and $P2X_4$ receptors in the vestibular system using genetically engineered mice and human patients with mutations will provide insight into the exact roles of purinergic receptors in the vestibular system for maintaining balance.

V. CONCLUSION

$P2X_2$ and $P2X_4$ receptors regulate ion transport in the utricular transitional cells and macula by ATP stimulation in excessive linear acceleration situations. The $P2X_2$ and $P2X_4$-mediated cation transport is likely to be involved in the protection of hair cell and modulation of signal transduction in hair cell during the event of excessive linear acceleration.

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ABSTRACT(IN KOREAN)

난형낭 전이세포와 평형반에서의 $P2X_2$와 $P2X_4$ 수용체 매개 양이온 흡수

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정준희

adenosine 5'-triphosphate (ATP)는 내이 상피세포에서 퓨린 수용체를 통한 이온 수송을 조절함으로써 내이 기능을 조절한다. 이 연구에서 선형 가속도 자극이 감지되는 쥐의 난형낭 평형반과 주변 전이세포에서의 퓨린 수용체 매개 양이온 수송을 조사하였다. ATP, adenosine 5'-diphosphate (ADP) , uridine 5'-triphosphate (UTP), uridine 5'-diphosphate (UDP) 중에서 ATP (100 μM)만 전이세포와 평형반에서 양이온 흡수 전류를 유도하였다. 이 전류는 gadolinium (100 μM)을 적용하였을 때 대부분 완전히 억제되었다. 양이온 흡수 전류에 대한 작용제 효능 순서는 전이세포와 평형반 모두에서 ATP > 3'-*O*-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP) >> α,β-methyleneadenosine 5'-triphosphate (αβmeATP)였고 ATP, bzATP, αβmeATP의 EC_{50} (절반의 최고 효과를 나타내는 농도) 값은 전이세포에서 27.2 μM, 43.9 μM, 34.5 μM였고 평형반에서 20.7 μM, 63.4 μM, 2014.1 μM였다 (αβmeATP의 EC_{50} 값은 αβmeATP의 낮은 효능으로 확실히 확인되지 않았다). ATP 유도 전류는 두 부위에서 suramin (100 μM), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) (10 μM), 그리고 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one

(5-BDBD) (5 μM)에 의해 부분적으로 차단되었고 PPADS + 5-BDBD에 의해 거의 완전히 차단되었다. 면역세포화학법에서 전이세포와 평형반의 지지세포에 $P2X_2$ 수용체가 분포한다는 것이 확인되었다. 그러나 $P2X_4$ 수용체는 면역세포화학법으로 평형반에서 발견되지 않았지만 그것의 messenger ribonucleic acid (mRNA) 발현이 확인되었다. 이 결과들은 ATP가 난형낭 전이세포와 평형반에서 $P2X_2$와 $P2X_4$ 수용체를 통해서 이온 흡수를 유도한다는 것을 나타낸다. $P2X_2$와 $P2X_4$ 매개 양이온 수송은 과도한 선형 가속도 조건에서 양이온 이동으로 유모세포의 양이온 부담을 줄여서 유모세포를 보호할 것으로 생각된다.

핵심되는 말 : 난형낭, 전이세포, 평형반, 수용체, 양이온