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INVITED REVIEW

Purinergic signaling in the peripheral vestibular system

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Abstract

The inner ear comprises the cochlea and vestibular system, which detect sound and acceleration stimulation, respectively. The function of the inner ear is regulated by ion transport activity among sensory epithelial cells, neuronal cells, non-sensory epithelial cells, and luminal fluid with a unique ionic composition of high [K^+] and low [Na^+], which enables normal hearing and balance maintenance. One of the important mechanisms regulating ion transport in the inner ear is purinergic signaling. Various purinergic receptors are distributed throughout inner ear epithelial cells and neuronal cells. To date, most studies have focused on the role of purinergic receptors in the cochlea, and few studies have examined these receptors in the vestibular system. As purinergic receptors play an important role in the cochlea, they would likely do the same in the vestibular system, which is fairly similar to the cochlea in cellular structure and function. Based on available studies performed to date, purinergic signaling is postulated to be involved in the regulation of ion homeostasis, protection of hair cells, otoconia formation, and regulation of electrical signaling from the sensory epithelium to vestibular neurons. In this review, the distribution and roles of purinergic receptors in the peripheral vestibular system are summarized and discussed.

Keywords Purinergic receptors · Inner ear · Vestibular system · Semicircular canal · Utricle · Saccule

Sung Huhn Kim graduated with an M.D. from Yonsei University College of Medicine and obtained a PhD degree from the Graduate School of Yonsei University. He completed postdoctoral training and worked as visiting scientist in Department of Anatomy and Physiology (P.I. Daniel C. Marcus and Philine Wangemann), Kansas State University. His main research interest is ion transport, homeostasis of the inner ear and related disorders. Recently, he investigated the pathological mechanism of mutations in ion channel genes in the peripheral vestibular system.

Introduction

The inner ear comprises cochlear and vestibular compartments. Both compartments detect mechanical stimuli and convert them to electrical signals that are transmitted to the central nervous system. Mechanical stimulation of the cochlea and vestibular system is sound and angular/linear acceleration. Both structures are composed of a luminal structure formed by a thin epithelial layer surrounded by an abluminal space enclosed by a hard bony capsule named temporal bone. The luminal space is called the endolymphatic space and filled with endolymphatic fluid composed of high [K^+] (~ 150 mM) and low [Na^+] (~ 1–10 mM), and the abluminal space is called the perilymphatic space and filled with fluid composed of high [Na^+] (~ 150 mM) and low [K^+] (~ 4 mM) [1]. The unique ionic milieu of endolymphatic fluid is important in converting mechanical stimulation to an electrical signal by hair cells in the cochlea and vestibular system. Fluid homeostasis and neuronal activities are regulated by various ion channels, transporters, exchangers, and signaling molecules in epithelial and neuronal cells in each compartment [1]. Purinergic receptors are some of the important regulators of these activities [2]. They consist

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of metabotropic (G protein–coupled receptors; P1 and P2Y receptors) and ionotropic receptors (ligand-gated ion channels; P2X receptors) and are regulated by purine nucleotides. Various purinergic receptors are distributed throughout the sensory and non-sensory epithelial cells and neuronal cells in the inner ear [2–6]. In the cochlea, many experimental studies and several excellent reviews have discussed purinergic receptors and signaling [3–6]; however, few studies have been conducted on the vestibular system. The vestibular system maintains body balance and shares similar cellular structures, functions, and extracellular environments with those in the cochlea. Based on the similarity between the two compartments, various purinergic receptors should be present in the vestibular system and play important roles, similar to the cochlea. In fact, the distribution and role of purinergic receptors identified in the vestibular system thus far are similar to the corresponding cell types in the cochlea; however, one would suspect that they would have developed to perform specific roles in different environments and in response to different stimuli. In this review, purinergic receptors and signaling in the vestibular system, their implications, and applicable future research areas are discussed in detail.

Anatomy and cellular components of the vestibular system

The vestibular system is composed of three semicircular canals with the proximal ampullary portions, utricle, and saccule (Fig. 1a). Each semicircular canal is located perpendicular to the other canals in three dimensions and detects angular acceleration from different stimulation angles [7]. Utricle and saccule detect horizontal and vertical linear acceleration stimulation, respectively [7]. The ampullary portion of the semicircular canal contains the crista ampullaris, consisting of neuroepithelium, a matrix of hair cells, and supporting cells, sitting atop a connective tissue stroma, called the cupula which extends to the roof of the ampulla (Fig. 1b). The utricle and saccule contain macula similar in structure to the crista ampullaris, consisting of neuroepithelium, a matrix of hair cells, and supporting cells, sitting atop a connective tissue stromal layer, called the otoconial membrane, and otoconia, tiny calcium carbonate crystals, on the stromal layer (Fig. 1c). The neuroepithelium is innervated by a separate nerve branch whose cell bodies are in Scarpa's ganglion of the vestibular nerve and which detects mechanical stimuli. Hair cells play a key role in detecting such stimulation. They are divided into two types, namely, type 1 and type 2 hair cells, according to morphology, type of synaptic nerve ending, and their action [8]. Type 1 hair cells have a flask shape and are innervated by a single calyx-type or both calyx- and bouton-type nerve endings. Type 2 hair cells have a cylindrical shape and are innervated by bouton-type nerve endings. Stereocilia are located on the apical membrane, a cuticular plate, of hair cells and are arranged like a staircase with the tallest cilia in the rank nearest the kinocilium. Hair cell stereocilia tilt toward or opposite direction of kinocilia through the movement of endolymphatic fluid during acceleration stimulation. The tilting movement of stereocilia to the kinocilium opens mechanosensitive non-selective cation channels through which K^+ enters the cytosol. This process evokes a change in the hair cell membrane potential, and consequently, Ca^{2+} enters through voltage-gated Ca^{2+} channels located at the basolateral surface [9]. Then, neurotransmitters are released from the basolateral surface of hair cells to the synaptic nerve ending and induce vestibular nerve depolarization. K^+ in hair cells escapes from cells through basolaterally located K^+ channels (molecular identities were not fully identified) to synaptic cleft and enters to calyx-type nerve endings through cation channels (a cyclic nucleotide-gated channel was proposed, but the molecular identity was not fully identified) in type 1 hair cells or to supporting cells through gap junctions in type 2 hair cells [8, 10, 11]. The tilting movement of stereocilia in opposite directions to the kinocilium induces closure of the mechanosensitive channels, and K^+ influx to hair cells decreases, which consequently reduces the neural firing of the vestibular nerve. Non-sensory epithelial cells extend from the sensory epithelium and form the luminal structure of the vestibular compartment. These cells are transitional cells, vestibular dark cells, and other cells. Transitional cells are distributed adjacent to the sensory epithelium and have a certain role in regulating endolymphatic ion homeostasis and neighboring cellular activities [12, 13]. Vestibular dark cells are distributed in the utricular roof epithelium, ampullary floor near the transitional cells, and common crus, a structure in which the superior and posterior semicircular canals join together. They play an important role in providing K^+ in the endolymphatic fluid through apically located KCNQ1/KCNE1 [1]. Other epithelial cells are distributed over the saccular roof epithelium, semicircular canal, and common crus. They are involved in ion homeostasis by transporting cations and anions such as Na^+ and Cl^- through various ion channels, transporters, and exchangers [14–16]. All epithelial cells have tight junctions that separate the endolymphatic and perilymphatic space [17].

Source of purine nucleotides in the vestibular system

The only identified purine nucleotide released to the endolymphatic space is adenosine triphosphate (ATP) in the cochlea. The sites of ATP release are presumed to be marginal cells of the stria vascularis and connexin hemichannels

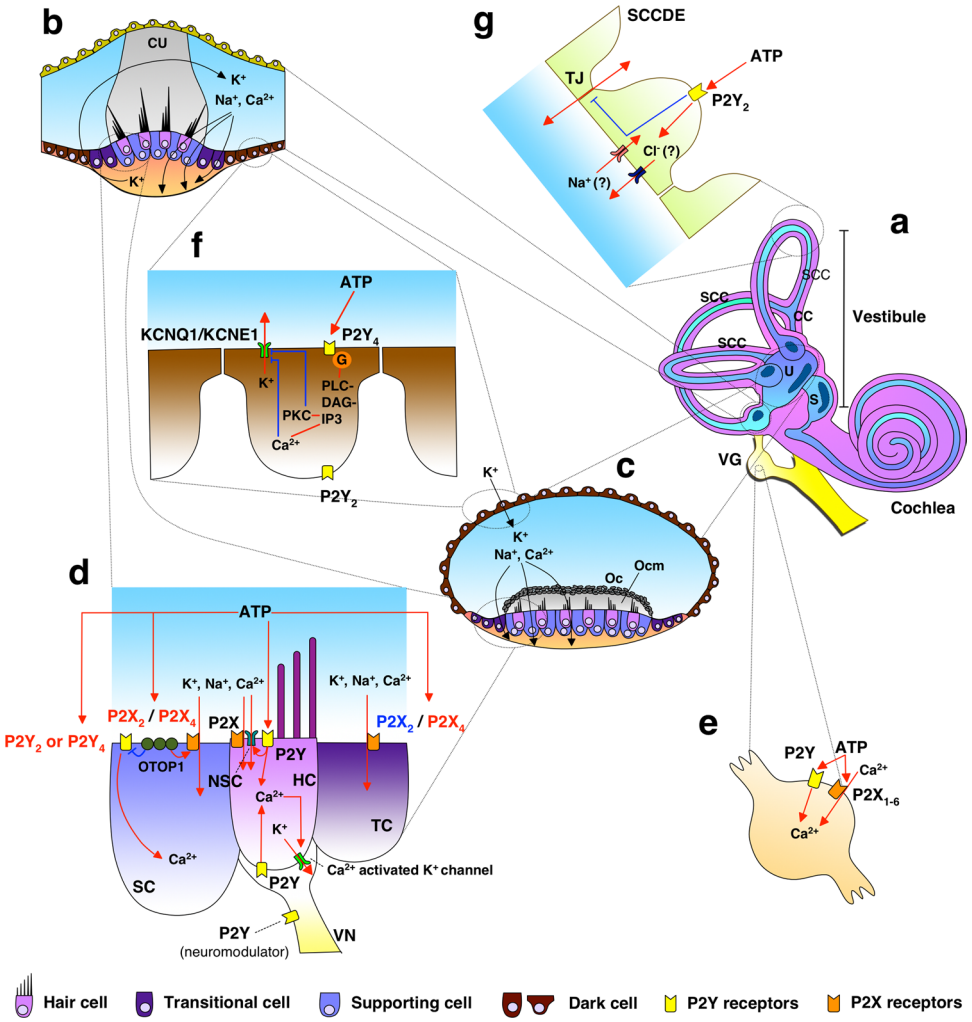

Fig. 1 Schematic of the functionally proven distribution of P2 receptors in the peripheral vestibular system. **a** Inner ear structure, which consists of cochlear and vestibular system. SCC, semicircular canal; CC, common crus; U, utricle; S, saccule; VG, vestibular ganglion. **b** Structure and cation transport by epithelial cells of the semicircular canal ampulla. CU, cupula. **c** Structure and cation transport by epithelial cells of the utricle. Oc, otoconia; Ocm, otoconial membrane. **d** ATP-induced cation transport and release of intracellular Ca^{2+} stores via purinergic receptors in supporting cells, hair cells, and transitional cells. In type 1 and type 2 hair cells, cations are absorbed by apically located P2X receptors or non-selective cation channels activated by P2 receptors. Apical P2 receptors (perhaps P2Y receptors) and basolateral P2Y receptors (type of hair cell was not identified) induce intracellular [Ca^{2+}] release. ATP induces efflux of intracellular K^+ absorbed through mechanosensitive non-selective cation channels on hair cell cilia, non-selective cation channels, and/or P2X receptors in apical membrane via basolaterally located Ca^{2+}-activated K^+ channels by inducing P2 receptor–mediated intracellular [Ca^{2+}] increase. In distal vestibular afferent nerve, P2Y receptors modulate neuronal firing rate. In the supporting cell, cations are absorbed apically through $P2X_2$ and $P2X_4$ receptors. Apical $P2Y_2$ or $P2Y_4$ receptors induce intracellular [Ca^{2+}] release. Otopetrin 1 (OTOP1) increases Ca^{2+} influx via OTOP1 itself and/or interacting P2X receptors or other proteins. OTOP1 inhibits the activity of $P2Y_2$ or $P2Y_4$ receptors and regulates intracellular [Ca^{2+}]. In transitional cells, cations are absorbed via $P2X_2$ and $P2X_4$ receptors. Red: transitional cells in the utricle, blue: transitional cells in the ampulla and utricle. **e** Intracellular Ca^{2+} release and Ca^{2+} influx in the vestibular ganglion. $P2X_{1\text{-}6}$ receptors may be involved in Ca^{2+} influx, but the specific type of P2X receptor was not identified. Intracellular Ca^{2+} release was likely to be mediated by P2Y receptors, but the type of P2Y receptors was not identified. **f** Regulation of K^+ efflux from vestibular dark cells by $P2Y_2$ and $P2Y_4$ receptors. K^+ efflux via KCNQ1/KCNE1 is presumed to be inhibited by apical $P2Y_4$ and basolateral $P2Y_2$ through the phosphorylation of the β-subunit of the apical K^+ channel by protein kinase C or direct inhibition by increased intracellular Ca^{2+} concentrations or by altered levels of membrane phosphatidylinositol-4,5-bisphosphate. PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate. **g** $P2Y_2$ regulation of paracellular ion transport in the semicircular canal duct epithelium. $P2Y_2$ receptors decrease the resistance of paracellular tight junctions and increase the permeability of ions. The receptor may be involved in apical Na^+ influx and Cl^- efflux, for which direct evidence was not provided. SCCDE, semicircular canal duct epithelium. The sky blue area on each figure represents endolymphatic space

in the supporting cells of the organ of Corti. In marginal cells, ATP was identified to be stored as vesicles close to the apical membrane by quinacrine staining and electron microscopy, but no direct evidence of ATP release in strial marginal cells has been reported [18]. An ex vivo study using a luciferin-luciferase bioluminescent assay in cochlear explants revealed that ATP was released by mechanical stimulation or in Ca^{2+}-free solution through gap junction hemichannels on the supporting cells of the organ of Corti [19]. The released ATP itself or its hydrolyzed and converted form ADP stimulates P2 receptors in cochlear epithelial cells. In the vestibular compartment, no evidence of purine nucleotide release has been reported. Vestibular dark cells and supporting cells share functional homology with cochlear strial marginal cells and supporting cells [20–22]. The distribution of ion channels, transporters, exchangers, and gap junctions is very similar between the cell types. The main role of vestibular dark cells is K^+ efflux into the endolymphatic space through apically located KCNQ1/KCNE1, similar to strial marginal cells [22]. Similar types of gap junctions are distributed over supporting cells, and various molecules and ions pass through them [20]. Considering the functional and structural homology of the cell types, ATP may be released from vestibular dark cells and supporting cells either as vesicles or through hemichannels. Recently, we observed that ATP was stained by quinacrine over vestibular dark cells and supporting cells, but not in other non-sensory epithelia. We also found that ATP was released by mechanical stimulation in ex vivo preparations, which was partially blocked by bafilomycin and nearly completely blocked by carbenoxolone (unpublished data). The mechanical stimulus in the vestibular system corresponding to sound stimulation in the cochlea is acceleration. Based on this observation, ATP is likely released upon acceleration stimulation and acts through purinergic receptors to regulate various cellular activities in the vestibular compartment.

Distribution of purinergic receptors in the vestibular system

Purinergic receptors were revealed to be distributed throughout the vestibular compartment by molecular and functional studies (Table 1 and Fig. 1). An early study in 1999 documented the expression of $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$ transcripts in whole rat vestibular organs at different developmental stages [23]. The expression of these transcripts disappeared at postnatal development day 16, except for $P2X_7$, in the study. The detailed localization of P2 receptors in each vestibular subcompartment and cell type was not identified in this study. In adult guinea pig vestibular hair cells, patch-clamp and intracellular $[Ca^{2+}]$ imaging using Fura-2 acetoxymethyl ester (AM) revealed the presence of P2 receptors, but the type was not identified [24]. ATP-evoked vestibular nerve firing caused by neurotransmitter release from hair cells via basolateral P2Y receptors and/or P2Y receptors in the distal vestibular nerve was reflected by ampullary nerve recordings in adult frogs, in which the type of receptor was not further identified [25]. In supporting cells, several studies have identified P2X and P2Y receptors in animal experiments. Expression of the $P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_5$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ transcripts was observed using RT-PCR in dissociated non-sensory utricular macular epithelial cell cultures from postnatal day 3 (P3) mice, and $P2Y_2$ and $P2Y_4$ were detected on the apical side of the cells in the macular epithelium of E18.5 mice using immunocytochemistry [26]. The function of P2Y receptors was determined by performing intracellular $[Ca^{2+}]$ imaging using Fura-2 AM in dissociated cells from the utricular macular in embryonic day 18.5 (E18.5) – P3 mice. In supporting cells of adult mouse ampulla and utricle, $P2X_2$ was detected using immunocytochemistry and in situ hybridization [27]. Furthermore, a recent study identified the presence and function of $P2X_2$ and $P2X_4$ at the apical region of adult mouse utricular supporting cells by measuring transepithelial currents and immunocytochemistry [12]. In adult gerbil and mouse ampullary and utricular transitional cells, the functional activity of apical $P2X_2$ was measured by electrophysiological and pharmacological experiments [12, 13]. In addition, $P2X_4$ was detected at the apical region of adult mouse utricular transitional cells in electrophysiological and pharmacological experiments, as well as using immunocytochemistry [12]. In vestibular dark cells, no definite evidence of the functional activity of P2X receptors was obtained, but most showed P2Y receptor activities. Animal experimental studies using adult gerbils and adult rats consistently showed the presence and activity of $P2Y_2$ on the apical side and $P2Y_4$ on the basolateral side of vestibular ampullary dark cells using electrophysiological and pharmacological experiments, RT-PCR, and immunocytochemistry [28–30]. The $P2Y_2$ receptor was also identified on the apical side of cultured neonatal rat semicircular canal epithelial cells using a gene array and electrophysiological and pharmacological experiments [31]. In adult rat vestibular ganglion cells, the expression of the $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_6$ proteins was detected using immunocytochemistry [32]. Transcripts encoding $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_6$ were also detected in the vestibular ganglion of newborn rats (P5–10) [33]. The ATP-induced current was detected using patch-clamp experiments in the study, but the type of P2X receptor was not functionally identified. Another study also detected ionotropic and metabotropic P2 receptor activity in the adult guinea pig vestibular ganglion by performing intracellular $[Ca^{2+}]$ imaging using Fura-2 AM but did not precisely identify the types of receptors [34]. A detailed discussion of the functional evidence and its implications for

Table 1 Purinergic receptor distribution in the peripheral vestibular system

Cell type	Subregion	Type of purinergic receptor	Location in cells	Animal	Experimental methods	References
Hair cell	Ampulla	P2	N/A	Guinea pig	Patch clamp, RT-PCR	[24]
	Ampulla	P2Y	Basolateral (and/or distal vestibular nerve)	Frog	Nerve recording	[25]
Supporting cell	Utricle	$P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_5$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$	Apical ($P2Y_2$ and $P2Y_4$)	Mouse	Intracellular $[Ca^{2+}]$ imaging, RT-PCR, immunocytochemistry	[26]
	Utricle	$P2X_2$, $P2X_4$	Apical	Mouse	Scanning vibrating electrode technique, immunocytochemistry	[12]
	Ampulla, utricle	$P2X_2$	Apical	Mouse	In situ hybridization, immunocytochemistry	[27]
Transitional cell	Ampulla, utricle	$P2X_2$, $P2X_4$ (utricle)	Apical	Mouse	Scanning vibrating electrode, immunocytochemistry (utricle)	[12, 13]
Dark cell	Ampulla, utricle	$P2Y_4$	Apical	Gerbil, rat	Micro-Ussing chamber (ampulla), immunocytochemistry (ampulla and utricle)	[28–30]
		$P2Y_2$	Basolateral	Rat	Immunocytochemistry	[30]
Ganglion cell	Vestibular ganglion	P2	N/A	Guinea pig	Intracellular Ca^{2+} imaging	[34]
		$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$	N/A	Rat	Immunocytochemistry	[32]
		$P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$	N/A	Rat	Patch clamp, RT-PCR	[33]
Whole vestibular system	N/A	$P2X_2$*, $P2X_3$*, $P2X_4$*, $P2X_7$	N/A	Rat	RT-PCR	[23]

N/A not available; *transcript expression disappeared on postnatal day 16

P2 receptors in the vestibular compartment will be described in the next sections.

Roles of purinergic receptors in vestibular hair cells and distal afferent nerves

In the cochlea, various P2X and P2Y receptors are distributed in outer and inner hair cells [3, 6]. These receptors regulate cochlear amplification and depolarization [3, 6]. In the vestibular system, several studies have investigated purinergic receptor activity in hair cells [24]. Patch-clamp experiments showed either 100 μM ATP-induced inward or outward currents in adult guinea pig crista type 1 and type 2 hair cells. The inward current likely occurred through non-selective cation channels with a reverse potential of − 35 mV, and the outward current was thought to originate from Ca^{2+}-activated K^+ channels, which are reported to be located at the basolateral surface in other studies [35–38]. After blocking the outward cation current with internal Cs^+ and external tetraethylammonium, an inward current with similar properties to these observations was detected. Intracellular $[Ca^{2+}]$ measurements with Fura-2 AM detected that ATP induced an increase in $[Ca^{2+}]$ in the cytosol of type 1 and type 2 hair cells. In the repeated experiment, the increase in $[Ca^{2+}]$ was much smaller. This observation suggested that ATP induced intracellular Ca^{2+} release. Based on the findings described above, ATP activates both inward and outward cation currents, and an increase in the intracellular $[Ca^{2+}]$ may be accomplished both through cation influx by P2X receptors (or other P2 receptor–activated non-selective cation channels) and P2Y receptor–mediated intracellular Ca^{2+} release (Fig. 1d). Also, ATP induces K^+ efflux through Ca^{2+}-activated K^+ channels by P2 receptor–mediated intracellular $[Ca^{2+}]$ increases. This process may be involved in the regulation

of depolarization and repolarization of vestibular hair cells. In other words, purinergic signaling regulates hair cell sensitivity and activity in response to a certain degree of acceleration stimulation or gravitational vector changes. However, no further studies have characterized P2 receptors in vestibular hair cells.

Aubert et al. [25] demonstrated the effect of purine agonist on distal afferent nerve firing and d.c. nerve potential using adult frog (*Rana pipiens*) ampulla. They prepared whole semicircular canals from the frog ex vivo with mutual separation of endolymphatic and perilymphatic compartments. In the preparation, mechanical stimulation could be applied through the endolymphatic space of semicircular canals via pressure changes in a syringe connected to the endolymphatic space, and electrical signals from the afferent nerve of ampulla were recorded with or without stimulation. Spontaneous firing of the afferent nerve was increased with the application of 10^{-12}–10^{-4} M ATP to perilymphatic space, and marked increases were achieved with the application of 10^{-3} M ATP. In contrast, d.c. nerve potential was decreased in a dose-dependent manner from 10^{-11} to 10^{-3} M ATP. Application of P2Y agonist 2-methylthio-ATP (2 MeSATP) resulted in a larger increase in spontaneous firing than that caused by ATP. However, d.c. nerve potential was not modified below or equal to 10^{-5} M 2MeSATP and was increased by 10^{-3} M 2MeSATP. During hair cell depolarization induced by applying pressure to the endolymphatic space, evoked nerve firing decreased at ATP concentrations from 10^{-12} to 10^{-4} M and further decreased at 10^{-3} M ATP. A dose–response relationship during the application of 2 MeSATP was similar to that with ATP application. The abrupt change with 10^{-3} M agonists might suggest the presence of more than two purinergic receptors or the breakdown product of ATP, probably adenosine, that might be responsible for a negative feedback on the ATP effect. When pressure to the endolymphatic space was applied to the opposite direction for hair cell repolarization, application of ATP-induced inhibition of nerve firing was much smaller in the same concentration range used in the above experiments. In contrast, the signal was enhanced by 2 MeSATP in a dose-dependent manner. The amplitude of d.c. nerve potential was reduced in a dose-dependent manner by the application of 10^{-3} M ATP. The amplitude of d.c. nerve potential in the inhibitory phase was not modified by 2 MeSATP. The breakdown product of ATP, as explained above, may also be responsible for the reduced response of ATP-induced firing rate during stimulation and hyperpolarization of d.c. nerve potential, which elicited an increased threshold of sensitivity of the afferent nerve fibers. Application of P2X agonist alpha, beta methylene ATP (αβmeATP) and beta, gamma methylene ATP did not show any effect in the above experimental conditions. The results of the study suggested the presence of P2Y receptors at the basolateral surface of hair cells and/or distal afferent nerves. These receptors may play a role as neuromodulators of vestibular function by regulating neurotransmitter release from hair cells and/or spontaneous and evoked responses of afferent nerves.

ATP (100 μM)-evoked Ca^{2+} transients have been observed in type 1 hair cells in utricular macula from P4–7 Gad2-Cre GCaMP5G transgenic mice, in which changes in intracellular [Ca^{2+}] fluorescent signals could be detected [39]. There were two cell groups: one showed strong Ca^{2+} transient signaling, and the other showed less Ca^{2+} transient signaling. The former was likely to be type 1 hair cells with calyces, and the latter was thought to be supporting cells. The type and role of purinergic receptors in type 1 hair cell of this early postnatal age was not identified. The findings in the supporting cells are discussed in "Roles of purinergic receptors in the non-sensory vestibular epithelium."

An indirect evidence for the existence of P2 receptors in vestibular hair cells was suggested by detecting ectonucleotide triphosphate diphosphohydrolase 6 (NTPDase 6) in the supranuclear region and hair bundle of adult rat ampullary hair cells and hair bundles of macular hair cells [40]. It is an intracellular enzyme with an enzymatic preference for GDP and UDP. Its main function is expected to be the regulation of nucleotide levels in cellular organelles. It may play a role in regulating P2 receptor signaling, sensory transduction, and maintenance of vestibular hair bundles, but the exact function of NTPDase 6 is still uncertain because GDP and UDP release into the endolymphatic space has not been identified.

Roles of purinergic receptors in the vestibular ganglion

$P2X_{1\text{-}6}$ receptors were detected in the adult rat vestibular ganglion using immunocytochemistry, as described above [32]. Two functional studies of P2 receptors have been reported to date. A study measuring intracellular [Ca^{2+}] using Fura-2 AM in the isolated adult guinea pig vestibular ganglion identified that ATP increased the [Ca^{2+}] in a dose-dependent manner when administered in concentrations ranging from 0.1 to 1000 μM [34]. The increase in [Ca^{2+}] was even detectable without extracellular Ca^{2+}, indicating that ATP induced Ca^{2+} release from the intracellular stores, which was likely to be accomplished by P2Y receptors. The increase in [Ca^{2+}] was inhibited by ~ 50% upon the application of the P2 receptor inhibitor suramin and reactive blue 2. Extracellular Ca^{2+} influx was thought to be accomplished through receptor-mediated Ca^{2+} channels but not by L-type Ca^{2+} channels because the ATP-induced increase in [Ca^{2+}] was inhibited by ~ 50% with the application of 100 μM La^{3+}

but not 50 μM nifedipine. Although it was not discussed in the study, Ca^{2+} influx was likely to be mediated by non-selective cation influx by the P2X receptor because La^{3+} partially inhibited the increase in [Ca^{2+}], in which the [Ca^{2+}] measured after inhibition was greater than that in Ca^{2+}-free solution (Fig. 1e). Types of P2 receptors and Ca^{2+} channels were not identified in this study. Another study provided more detailed electrophysiological properties of P2 receptors in newborn rat vestibular ganglia [33]. In this study, 100 μM ATP induced an inward current with a decaying time constant of 2–4 s, EC_{50} of 11.0 μM, Hill coefficient of 0.82, and desensitization properties. This decaying profile of ATP-induced current corresponds to the value between rapidly desensitizing ($P2X_1$ and $P2X_3$) and slowly desensitizing subgroups ($P2X_2$ and $P2X_4$) of P2X receptors. Therefore, the authors postulated that heterogeneous P2X receptors were distributed over the vestibular ganglion. The order of purine agonist potency was ATP > αβmeATP > ADP. The current was partially inhibited by 100 μM suramin and completely abolished in Na^{+}-free NMDG solution, but was not changed at acidic pH. These electrophysiological and pharmacological profiles represented the heterogeneous involvement of P2X receptors but did not precisely define the type of receptors (Fig. 1e). These properties are very similar to those observed in the spiral ganglion of the cochlea. The main difference between the cochlear spiral ganglion and vestibular ganglion was the insensitivity of ATP-induced current to acidification in the vestibular ganglion. The sensitivity to acidification reflects that P2X receptors have a multimeric configuration [41–43], and the absence of a response to acidification in the vestibular ganglion represents the homomeric configuration of P2X receptors. P2Y receptors were functionally identified in the cochlear spiral ganglion, but the presence of P2Y was not confirmed in this experiment [44]. Because vesicular ATP was reported to be co-stored and co-released in peripheral nerves with acetylcholine or noradrenalin [45], the two studies described above indicate that ATP may act as a neurotransmitter or neuromodulator and that P2X receptors are likely to be involved in the modulation of neural excitability in vestibular neurons.

Roles of purinergic receptors in the non-sensory vestibular epithelium

Purinergic receptors were detected in vestibular transitional cells, supporting cells, dark cells, and semicircular canal epithelium in animal experiments (Table 1). In adult gerbil ampullary and adult mouse utricular transitional cells, ATP-induced inward currents were observed using the scanning vibrating electrode technique, which measures transepithelial short-circuit currents by placing platinum-iridium wire microelectrodes with a platinum ball at the tip of the electrodes vibrating at a frequency of 400–700 Hz close (~ 10 μM) to the epithelial surface [12, 13]. The electrophysiological and pharmacological characteristics revealed by the experiments suggested the presence of the $P2X_2$ receptor. The order of purinergic agonist potency in the transitional cell area was ATP > 2'- and 3'O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (bzATP) > > αβmeATP and ADP, UTP, or UDP had no effect. ATP and bzATP were full agonists, whereas αβmeATP was a partial agonist (EC_{50} = 180, 897, and 16,434 μM for ATP, bzATP, and αβmeATP in the ampullary transitional cells, respectively; EC_{50} = 24, 145, and 167 μM for ATP, bzATP, and αβmeATP in the utricular transitional cells, respectively). The current was inhibited by ~ 80% and ~ 50% by suramin (100 μM) in ampullary and utricular transitional cells. Additionally, the ATP-induced current was inhibited by ~ 50% by 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD, 5 μM) and nearly completely inhibited by the mixture of pyridoxal-phosphate-6-azo-(benzene-2,4-disulfonic acid) (PPADS, 10 μM) and 5-BDBD in utricular transitional cells, which reflected the additional presence of $P2X_4$ receptors except $P2X_2$ receptors. These electrophysiological and pharmacological properties of utricular transitional cells were similar to those in utricular macula, where the EC_{50} values of ATP, bzATP, and αβmeATP were 29, 144, and 86 μM (partial effect), respectively, and the current was nearly fully inhibited by the same concentrations of PPADS and 5-BDBD. The simultaneous expression of $P2X_2$ and $P2X_4$ receptors was likely on transitional cells and supporting cells of the utricle, and immunofluorescent staining demonstrated the apical location of P2X2 and P2X4 receptors in those cells. In the supporting cells of the utricle, P2Y receptors might be involved in the inward current because a small, slowly rising current was also observed in some experiments. The aforementioned findings in ampullary transitional cells were similar to those in outer sulcus cells of the cochlea in which only $P2X_2$ receptors were identified by electrophysiological and pharmacological experiments [13]. The ATP-induced inward current in outer sulcus cells was proposed to provide a cation shunt that protects cochlear hair cells during noise stimulation by attenuating the cation burden. Similarly, vestibular transitional cells and supporting cells are likely to provide shunts during excessive acceleration stimulation, which can occur during sports driving, traffic accidents, whiplash injuries, aviation, and space flight, to protect vestibular hair cells (Fig. 1b and d).

Another study suggested the role of purinergic receptors in vestibular supporting cells for the formation and maintenance of otoconia, which are calcium carbonate crystals above the sensory epithelium of utricle and saccule [26]. Otoconia are important in detecting the linear acceleration and gravitational changes in the utricle and saccule. Otopetrin 1 (OTOP1), a multi-transmembrane domain

protein expressed at the apical region of supporting cells, has been reported to be essential in otoconia formation because *Otop1* knockout mice show head tilt, an inability to swim, and otoconia agenesis [46]. UTP (100 μM) and ATP (100 μM) increased intracellular [Ca^{2+}] in dissociated supporting cells from utricles of E18.5–P3 wild-type and *Otop1* knockout mice. The increase in [Ca^{2+}] showed a peak response and then a substantial decrease in non-OTOP1 expressing dissociated supporting cells of knockout mice, but the ATP-induced [Ca^{2+}] peak was much smaller and relatively sustained in OTOP1 expressing supporting cells of the wild-type mice. After removing intracellular Ca^{2+} with bisphenol, a difference was not observed. The response was not induced by ADP or UDP. This finding indicated the involvement of $P2Y_2$ or $P2Y_4$ receptors in increasing intracellular Ca^{2+} release from inositol-1,4,5-triphosphate-sensitive intracellular stores, and OTOP1 suppressed intracellular [Ca^{2+}] release by inhibiting $P2Y_2$ or $P2Y_4$ receptors (Fig. 1d). Ca^{2+} was suggested to be absorbed from an extracellular bath solution (140 mM NaCl, 5.4 mM KCl, 1 mM NaH2PO4, 1.8 mM CaCl2, 1 mM MgSO4,12 mM HEPES, and 5.5 mM D-glucose, pH 7.4 ± 0.1) used in the study by ATP stimulation through an interaction with P2X receptors and/or through OTOP1 itself or other proteins because the intracellular [Ca^{2+}] decay after ATP application in Ca^{2+}-free solution was steeper (Fig. 1d). The authors suggested that OTOP1 plays a role in otoconia formation and maintenance by regulating intracellular and extracellular [Ca^{2+}] through purinergic receptors.

A recent study by Rabbitt and Holman [39] revealed 100 μM ATP-evoked Ca^{2+} transient signals in supporting cells of the anterior and posterior semicircular canal eminentia cruciata and macula from early postnatal days (P4–7) in Gad2-Cre GCaMP5G transgenic mice Responses to ATP, such as latency and increasing and decreasing signal patterns, varied. The types and locations of purinergic receptors in the supporting cells were not identified in the study. The intercellular propagation of Ca^{2+} transient signals between vestibular supporting cells was modest when compared to that between inner border cells and adjacent cells in the cochlea (2 μm-s^{-1}, decaying to zero after ~ 3 cells). The Ca^{2+} transient signals in the cochlea are thought to play a role in the development of tonotopy, and the different properties of Ca^{2+} transient signals in vestibular supporting cells, compared to that in the cochlear, may suggest that purinergic signaling in the early postnatal developing vestibular system has different roles with that in the cochlea.

In vestibular dark cells, purinergic receptors are mainly involved in regulating apical K^+ efflux (Fig. 1f). The apical K^+ outward current decreased after the apical application of 100 and 10 μM ATP and UTP to adult gerbil vestibular dark cells in a micro-Ussing chamber experiment [28, 29]. The order of purine agonist potency for current inhibition was UTP > ATP > diadenosine tetraphosphate > > UDP (EC_{50} value of each agonist = 0.18, 5.5, and 70 μM, respectively). Application of an external Ca^{2+}-free solution, suramin, or PPADS did not exert any effect. These electrophysiological and pharmacological profiles suggested the presence of $P2Y_2$ and $P2Y_4$ receptors. Basolateral application of purine agonists showed an initial small increase in I_{sc} followed by a decrease in I_{sc}. The order of purine agonist potency was 2-methylthioadenosine 5′-triphosphate (2-meS-ATP) > ATP > > UTP = αβmeATP = adenosine and adenosine 5′-O-(3-thiotriphosphate) (ATPγS) for stimulation and ATPγS > 2-meS-ATP > ATP > UTP > αβmeATP. The former and latter profiles represent the presence of P2Y receptors and simultaneous P2U and P2Y receptors. Immunocytochemistry revealed that P2Y4 and P2Y2 receptors were located on the apical and basolateral sides, respectively [30]. Unsurprisingly, these characteristics of vestibular dark cells were generally similar to those of strial marginal cells because both cell types share similar functional homology in K^+ efflux [22]. K^+ efflux is likely modulated by the phosphorylation of the β-subunit of the apical K^+ channel by protein kinase C or direct inhibition by increased intracellular Ca^{2+} concentrations or by altered levels of membrane phosphatidylinositol-4,5-bisphosphate. A tempting speculation is that P2Y receptors in vestibular dark cells also contribute to the protection of hair cells and the regulation of inner ear homeostasis [2]. Excessive mechanical stimulation may induce ATP release from vestibular epithelial cells, thereby decreasing K^+ efflux by vestibular dark cells through $P2Y_2$ and $P2Y_4$ receptors. Along with the function of $P2X_2$ and $P2X_4$ receptors in transitional and supporting cells, this may be an additional mechanism with which to reduce cation burden in hair cells and/or facilitate fine adjustment of hair cell function.

In neonatal rat cultured semicircular canal epithelium, basolateral application of 100 μM ATP and UTP elicited biphasic changes: it induced initial transient transepithelial voltage peak increases, without any changes in transepithelial resistance changes, and delayed (~ 1–2 min) decreases in transepithelial resistance, without changes in I_{sc}, in Ussing chamber experiments [31]. Based on the gene array data reported in the study, the $P2Y_2$ receptor regulates cation influx (or anion efflux) and the paracellular pathway of ion transport (Fig. 1g).

To date, no functional studies on purinergic signaling in saccules have been conducted. Although the structure and role of the saccular macula are similar to those of the utricle, dark cells are not present in the saccule. Therefore, differences in the distribution and role of purinergic receptors in the saccule should occur, especially in the nonsensory epithelium. These should be identified in future studies through detailed molecular biological and functional experiments.

The purinergic receptors in the non-sensory epithelium are presumed to modulate ion transport as a protective mechanism for hair cells or indirectly modulate hair cell function by regulating the ionic milieu of the environment or neighboring cells.

Future implications for the research on purinergic signaling in the vestibular system

To date, $P2X_2$ is the only purinergic receptor that has been identified to have a role in the pathological phenotype of the inner ear in humans. Several *P2RX2* mutations that result in sensorineural hearing loss have been reported [47–50] [44–47]. These mutations are inherited in an autosomal dominant pattern and result in progressive bilateral sensorineural hearing loss to various degrees, depending on the mutation type. In addition, *P2rx2* knockout mice showed greater hearing loss levels after exposure to high-level noise than wild-type mice [50, 51]. $P2X_2$ receptors were identified in outer hair cells, supporting cells, outer sulcus cells, and Reissner's membrane in animal models, and sensorineural hearing loss might result from complicated pathological mechanisms [6]. One of the potent mechanisms underlying hearing loss and noise sensitivity is likely to be the impairment of cation shunting by $P2X_2$ receptors through supporting cells and outer sulcus cells. A similar mechanism also exists in vestibular organs through vestibular transitional cells and supporting cells; however, the vestibular phenotype of $P2X_2$ receptor dysfunction has not been reported in humans. Recently, an experiment using *P2rx2* knockout mice showed partially decreased vestibular function, as measured using the vestibulo-ocular reflex [27]. This may mean that vestibular dysfunction can be induced by usual mechanical stimulation in daily activity in *P2rx2* knockout mice without excessive mechanical stimuli in the experimental setting. The degree of vestibular dysfunction may be much worse after acute or chronic exposure to acceleration stimulation in patients or animals with pathological *P2rx2* mutations. Although bilateral minimal vestibular loss is not apparent due to substitution of other sensory systems or habituation, progressive bilateral severe vestibular dysfunction results in difficulty in maintaining body balance, which causes restricted body movement and is a high-risk factor for falling. The distribution and function of purinergic receptors in the cochlea have been intensively investigated, but research on the vestibular system is still sparse. Both organs share substantial functional homology in detecting stimulation and signal transduction, although purinergic receptors and their signaling pathways appear to differ, as do their specific roles in the vestibular system to maintain normal balance function. Purinergic signaling via various purinergic receptors has specific roles throughout multiple organs. ATP act as a neurotransmitter, gliotransmitter, and paracrine factor in special sensory organs, including the inner ear, retina, olfactory epithelium, and taste buds [52–54]. Purinergic signals mediate sensory signal transduction, modulate the regulation of synaptic transmitter release, and regulate bidirectional neuron-glial interactions. In addition, purinergic signaling is involved in the control of vascular tones to control blood supply, extracellular ion homeostasis, apoptosis, and progenitor proliferation [52]. Although stimuli and extracellular environments differ among sensory organs, purinergic signaling seems to have considerable commonality among the sensory organs in that it is involved in the transduction of stimuli, information processing, and crosstalk between neurons and supporting cells. In general, P2X receptors, especially $P2X_2$ and $P2X_7$, are frequently involved in the modulation of sensory neuronal actions, and $P2Y_1$ and $P2Y_2$ receptors are characteristic of pathways to and among supporting and glial cells [52–54]. However, purinergic signaling also has specialized functions with different requirements for each organ: For instance, $P2X_7$ receptors are reported to be expressed by nearly every type of retinal neuron. Researchers have indicated that they are distributed at rat photoreceptor terminals near ribbon synapses, horizontal cells, and amacrine cells, while $P2X_2$ receptors are localized to GABAergic amacrine cells and ganglion cells [52]. They play roles in neurotransmission and neuromodulation in the retinal visual pathway. In the inner ear, $P2X_2$ receptors were found to be distributed over cochlear hair cells, supporting cells, outer sulcus cells, vestibular transitional cells, and vestibular supporting cells [2]. Here, they play roles in hair cell amplification and depolarization, and provide cation shunt by non-sensory epithelial cells against mechanical overstimulation of hair cells. In spiral and Scarpa's ganglion of the inner ear, $P2X_2$ receptors modulate neuronal activity. $P2X_7$ receptors were also found to be expressed in rat cochlear hair cells, supporting cells, spiral ganglion, and vestibular supporting cells; however, the role of the receptors in the inner ear remains uncertain [52]. Even in the inner ear, the role of purinergic signaling may differ between the cochlea and vestibular system. In support thereof, Rabbitt and Holman [39] showed that Ca^{2+} transients linked to ATP signaling differed between cochlea and vestibular epithelia at early postnatal stages and were much weaker in vestibular cells, as described in "Source of purine nucleotides in the vestibular system," "Roles of purinergic receptors in vestibular hair cells and distal afferent nerves," and "Roles of purinergic receptors in the non-sensory vestibular epithelium", indicating that purinergic signaling would have specialized functions in accordance with specific requirements of each structure. In the vestibular system, purinergic signaling appears to be involved in the regulation of sensory transduction in hair cells and neurons of the ampullary crest of

the semicircular canal and macula of otolithic organs, while in non-sensory epithelium, it would have roles in the regulation of ion homeostasis regulation. Also, in the non-sensory epithelium, purinergic signaling would differ in vestibular dark cells, supporting cells, and other non-sensory epithelial cells, as described in the above sections. The specific cell types are localized in the limited area of each structure of the vestibular system. Hair cells and supporting cells are localized only in the ampullary crest and macula; vestibular dark cells are localized in the ampulla, utricular roof, and common crus, but not in the saccule; and other simple cuboidal non-sensory epithelial cells are localized in the semicircular canal, saccular roof, and common crus. As such, purinergic signaling might have different roles in individual structures of the vestibular system and may be organized accordingly to regulate efficient sensory transmission. This should be elucidated through future studies. For this, the distribution of purinergic receptors and their types in the vestibular system should be defined in detail, and functional validation should be performed. Recent advances in creating conditional knockout or mutant mice enable us to identify the detailed role of each type of receptor in the vestibular organ. Additionally, the method for chronic and acute mechanical stimulation of vestibular organs should be established and standardized for further validation of the role of purinergic receptors in each mouse strain with gene mutations as a noise-induced hearing loss mouse model. Many unknown roles of purinergic receptors and signaling in the vestibular system remain to be identified. Future interest and efforts of researchers will contribute to elucidating the mechanism of purinergic signaling in the maintenance of human balance by peripheral vestibular organs and their connection to the central nervous system.

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Declarations

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