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Purinergic signaling in the inner ear

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Abstract

Epithelial cells of the inner ear coordinate their ion transport activity through a number of mechanisms. One important mechanism is the autocrine and paracrine signaling among neighboring cells in the ear via nucleotides, such as adenosine, ATP and UTP. This review summarizes observations on the release, detection and degradation of nucleotides by epithelial cells of the inner ear. Purinergic signaling is thought to be important for endolymph ion homeostasis and for protection from over stimulation.

Keywords

cochlea; vestibular labyrinth; purinergic receptor; stria vascularis; marginal cell; vestibular dark cell; nucleotide release; ATP; UTP; ecto-nucleotidase

Introduction

The inner ear consists of the peripheral organs of hearing (cochlea) and balance (vestibular labyrinth) (Fig. 1). These organs are comprised of epithelial cells that need to coordinate their transepithelial transport activity in both the presence and absence of mechanical stimuli, as well as in the face of systemic and local environmental challenges. Many of these cells do not have cell-to-cell gap junction communication (Kikuchi et al., 1994; Kikuchi et al., 1995) and therefore must rely on other signals to coordinate their activity. Nucleotides and their purinergic receptors appear to form the basis of important paracrine and autocrine communication systems in the inner ear. Hormonal signaling pathways consist of the controlled release of agonist from a cellular source, the receptors for the agonist and a termination mechanism that degrades or otherwise inactivates the agonist. There is evidence for the existence of all of these elements in purinergic signaling pathways in the inner ear. Further, there is evidence that the cochlear purinergic signaling systems are activated by acoustic stimulation.

Sources of agonists

Three possible sites have been identified for the sources of adenosine triphosphate (ATP) in the inner ear; 1) strial marginal cells and vestibular dark cells, 2) connexin hemichannels in cochlear supporting cells, 3) cells in the organ of Corti. White et al. (1995) have demonstrated

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vesicles in close proximity to the apical membrane of marginal cells that reside in the stria vascularis. These vesicles stained with quinacrine, an acridine compound that binds to adenine nucleotides, particularly ATP (White et al., 1995). The morphological study by electron microscopy revealed that most of the vesicular structures were located within a layer 2-4 μm from the luminal or endolymphatic surface (White et al., 1995). In the lateral wall, the vesicular fraction of membrane-bound ATP was significantly greater (2.02 nmol ATP/mg protein) than the amount in the organ of Corti (0.69 nmol ATP/mg protein) (Munoz et al., 2001). There is as yet no direct evidence in vestibular dark cells; however, considering the functional homology between strial marginal cells and vestibular dark cells (Wangemann, 1995), it would be a likely site for ATP release in the vestibular labyrinth. Ca^{2+} -dependent release of ATP has also been observed in the organ of Corti *in vitro* measured with a luciferase bioluminescence assay (Wangemann, 1996), although no vesicular storage of ATP has been identified there. Recently, connexin gap junctional hemichannels in supporting cells of the organ of Corti have been proposed as a possible source of ATP in the cochlea (Zhao et al., 2005). Gap junctions provide a diffusion pathway between adjacent cells by alignment of hemichannels composed of connexin subunits in each adjacent cell to form an open barrel structure connecting the cytosol of the two cells. However, the individual hemichannels can also occur in the plasma membrane at sites that do not connect to adjacent cells and therefore can provide a diffusion pathway between the cytosol and the extracellular fluid. Hemichannels can pass many small molecules up to about 1 kDa, including nucleotides. Isoform-specific selectivity of hemichannels has been described (Ayad et al., 2006; Zhao, 2005).

In the mammalian inner ear, connexins are expressed extensively in nonsensory supporting cells around cochlear and vestibular hair cells, but not in hair cells (Kikuchi et al., 1994; Kikuchi et al., 1995; Kikuchi et al., 2000). The connexin hemichannels also reside in the nonjunctional plasma membrane surfaces (Kikuchi et al., 1995). Zhao et al. demonstrated that ATP release could be increased 3 to 5-fold from the isolated cochlea, exclusive of the lateral wall, by a reduction of extracellular Ca^{2+} or an increase in membrane stress using hypotonic extracellular solution to increase cell volume (maneuvers known to increase fractional open time of connexin hemichannels), and the increase could be prevented by gap junction blockers (Zhao et al., 2005). Uridine triphosphate (UTP) and ATP are released from many epithelial cells via exocytosis, anion channels, and via as-yet-unidentified pathways in response to osmotic and mechanical stress (Hisadome et al., 2002; Homolya et al., 2000; Lazarowski et al., 1997; Schwiebert and Zsembery, 2003). However, UTP release has not yet been demonstrated in the inner ear.

Receptor distribution

Purinergic receptors occur as ionotropic (P2X) and metabotropic (P1 and P2Y) receptors, in several isoforms. The ionotropic receptors are ligand- (purine-) gated ion channels (cation non-selective) that have no intermediate signaling between the agonist and the effector. On the other hand, the metabotropic receptors are coupled via heterotrimeric G proteins in the plasma membrane to phospholipase C (PLC), which generates 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (InsP3). DAG leads via one branch of the pathway to activation and translocation of protein kinase C (PKC) from the cytosol to the plasma membrane and InsP3 leads via a second branch of the pathway to elevation of cytosolic $[\text{Ca}^{2+}]$. The effectors respond to either the activated PKC or elevated $[\text{Ca}^{2+}]$.

Apical (endolymphatic side) receptors

The primary P2X receptor subtype found in cells lining the cochlear duct is the P2X₂ receptor (Xiang et al., 1999). P2X receptors in the apical membranes of cells lining the cochlear duct appear to mediate a protective decrease in the electrical driving force for acoustic transduction via a reduction in transepithelial resistance of the cochlear duct (Thorne et al., 2004). These

receptors are clearly located on the apical membrane of cochlear duct cells since this reduction in resistance occurs upon injection of ATP into the lumen (Thorne et al., 2004). There is functional evidence for the presence of these channels on the apical membrane of Reissner's membrane (King et al., 1998), outer hair cells (Housley et al., 1992), outer sulcus cells and vestibular transitional cells (Lee et al., 2001) (Fig. 1A, C).

Munoz et al have shown that noise elicits an increase in ATP concentration in endolymph (Munoz et al., 2001). Further, they found that when ATP is injected into scala media it causes a rapid and reversible fall in the endocochlear potential and a concomitant decrease in hearing sensitivity (increase in compound action potential thresholds) (Munoz et al., 1999; Munoz et al., 1995). They recently have shown that this ATP-induced fall in endocochlear potential can be attributed to a shunt across the cochlear partition (measured as a fall in cochlear partition resistance) arising from a cation (principally K^+) flux through ATP-gated ion channels (P2X receptors) present on the endolymphatic surface of epithelial cells lining scala media (Thorne et al., 2004). This is blocked by the P2X receptor-selective antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) injected into scala media. Sustained noise exposure (100 – 120 dB SPL, white noise, over 6 hours) up-regulates expression of P2X₂ and increases ATP-stimulated currents in the outer hair cells (Wang et al., 2003). They conclude that this ATP-mediated shunt of the endocochlear potential represents an autocrine and/or paracrine regulatory control process, which provides long-term homeostatic control of cochlear sensitivity and protection from acoustic damage.

The outer sulcus cells and vestibular transitional cells absorb cations via two kinds of nonselective cation channels at the apical membrane (Fig. 1D): ATP-sensitive (P2X₂) (Lee et al., 2001) and ATP-insensitive channels (Chiba and Marcus, 2000). The activation of P2X₂ leads to open ATP-sensitive nonselective cation channels (Lee et al., 2001), a process thought to protect the ear from overstimulation (above). The ATP-insensitive channel helps maintain the low Na^+ and Ca^{2+} levels of endolymph (Marcus and Chiba, 1999). The basolateral K^+ conductance establishes the driving force for cation absorption (Chiba and Marcus, 2001). Na^+ is removed across the basolateral membrane by Na^+ , K^+ -ATPase (Chiba and Marcus, 2001). Reissner's membrane also has P2X₂ receptors on the apical membrane, but these operate in parallel with Na^+ -selective epithelial sodium channels in the apical membrane (Lee and Marcus, 2003) rather than nonselective cation channels. Recent findings indicated that P2Y₄ receptors are expressed in developing outer sulcus cells, and then down-regulated in the adult (Lee et al., 2007).

Homeostasis of the luminal fluid composition is also controlled by P2Y₄ receptors in the apical membrane of strial marginal cells and vestibular dark cells of rodents (gerbil and rat) (Liu et al., 1995; Marcus et al., 2005; Marcus and Scofield, 2001; Marcus et al., 1998; Sage and Marcus, 2002). Activation of these receptors decreases the rate of K^+ secretion, which is mediated by the constellation of transporters illustrated in Fig. 1B. K^+ is taken up across the basolateral membrane of the strial marginal cells and vestibular dark cells by the Na^+ , K^+ -ATPase ("Na⁺-pump") and the Na^+ , K^+ , 2Cl⁻ cotransporter (NKCC1). Na^+ carried into the cell on the cotransporter is removed by the Na⁺-pump and Cl⁻ carried into the cell on the cotransporter leaves by passive diffusion via a large Cl⁻ conductance (ClC-K/Barttin) in the basolateral membrane. K^+ taken up by both the Na⁺-pump and the cotransporter is secreted across the apical membrane by diffusion via KCNQ1/KCNE1 K^+ channels.

K^+ secretion is thought to be modulated by at least three purinergic pathways in rodent: 1) phosphorylation of the β -subunit of the apical K^+ channel by protein kinase C (PKC) (Marcus et al., 1998; Marcus et al., 1997); 2) inhibition directly by increased intracellular Ca^{2+} (Shen and Marcus, 1998) and 3) by altered levels of membrane phosphatidylinositol-4,5-bisphosphate (PIP₂) levels (Cho et al., 2002; Loussouarn et al., 2003). First, the ATP-sensitive

short circuit current (a manifestation of K^+ secretion) of the strial marginal cells and vestibular dark cells was shown to be mediated via PKC, which was consistent with prior reports that the apical K^+ channel (KCNQ1/KCNE1) activity is inhibited by phosphorylation of KCNE1, the β -subunit of the channel (Cho et al., 2002; Loussouarn et al., 2003). By contrast, guinea pig ion transport, as reflected by cochlear voltages, does not respond to luminal UTP and therefore is not likely regulated by P2Y₄ or P2Y₂ receptors (Munoz et al., 1999). This apparent discrepancy is likely due to the absence of a PKC phosphorylation consensus site sequence in this species (Marcus et al., 1998). It was demonstrated that the introduction of ATP into the endolymph of the guinea pig decreased the endocochlear potential and the cochlear partition resistance. However, these decreases in voltage and resistance were shown to be due to activation of P2X₂ receptors, rather than P2Y₄ receptors (Thorne et al., 2004).

Second, KCNQ1/KCNE1 K^+ channels in the strial marginal cells were shown to be inhibited by divalent ions, including Ca^{2+} (Shen and Marcus, 1998). The increase in cytosolic Ca^{2+} levels in response to apical ATP could therefore act directly on the channel activity.

Third, the decrease in K^+ secretion by luminal ATP or UTP is maintained for at least 15 min in the continued presence of agonist (Marcus et al., 2005) although both cloned P2Y₂ and cloned P2Y₄ purinergic receptors have been reported to undergo desensitization within 5-15 min (Otero et al., 2000; Robaye et al., 1997). The most likely explanation for the apparent lack of desensitization (sustained decrease) of K^+ secretion in the inner ear is based on recent findings that activation of receptors (such as P2Y₄) coupled to G_{q/11} deplete PIP₂ (Cho et al., 2002) and that KCNQ1/KCNE1 K^+ channel activity is reduced by depletion of plasma membrane PIP₂ (Loussouarn et al., 2003). PIP₂ (phosphatidylinositol-4,5-bisphosphate) is a plasma membrane phospholipid that is normally a component of a second-messenger signaling cascade that results in the release of IP₃ to the cytosol and release of Ca^{2+} from endoplasmic reticulum stores via IP₃ receptors. Stimulation of such a signaling pathway would therefore deplete PIP₂ in the plasma membrane when it is converted to IP₃.

Interestingly, the depletion of PIP₂ might be associated with the involvement of myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS is the major PKC substrate in most mammalian cells (McLaughlin and Murray, 2005). It was recently shown that MARCKS acts as a reversible PIP₂ source in the plasma membrane, by electrostatically sequestering the negatively-charged PIP₂. When the cytosolic [Ca^{2+}] increases, Ca/Calmodulin binds to MARCKS with high affinity and pulls MARCKS off the membrane, releasing three PIP₂ molecules (McLaughlin and Murray, 2005).

Thus, the pathways from P2Y₄ stimulation converge (Figure 1): 1) G protein-PLC activation leads to consumption of membrane PIP₂, with the consequent reduction in K^+ channel activity; 2) the DAG-PKC path decreases K^+ channel activity directly via phosphorylation of the channel; and 3) the IP₃/ Ca^{2+} path decreases channel activity directly via the effect of Ca^{2+} on channel activity. The IP₃/ Ca^{2+} path also provides some recovery from PIP₂ depletion via Ca^{2+} /calmodulin-MARCKS.

Basolateral (perilymphatic side) receptors

Multiple P2 receptors in the basolateral membrane of the strial marginal cells and vestibular dark cells are known to contribute to homeostasis of the luminal fluid composition (Liu et al., 1995; Sage and Marcus, 2002). The ionic current produced across these epithelia is modulated in a biphasic manner by basolateral purinergic agonists. The receptor subtypes involved were tentatively identified as P2Y₂ (Sage and Marcus, 2002) and P2Y₁, although a P2X receptor has not been ruled out (Liu et al., 1995). Other evidence points to purinergic signaling in the organ of Corti; these P2 receptors do not likely contribute significantly to homeostasis of the luminal fluid composition but may influence the basolateral microenvironment of the inner

hair cells (Dulon et al., 1991; Sugasawa et al., 1996), Deiters' cells, and Hensen's cells (Dulon et al., 1993). The sidedness of the location of the receptors in these cells is not positively established. The receptors on the Deiters' cells are likely P2Y and the receptors on the Hensen's cells are likely P2X, as evidenced by the involvement of internal Ca^{2+} stores in the former but not the latter cells.

Termination

Extracellular nucleotides can be inactivated by hydrolysis via ectonucleotidases located at the cell membrane. These include the E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family, the E-NPP (ectonucleotide pyrophosphatase/phosphodiesterase) family, ecto-5'-nucleotidase, and alkaline phosphatases (Zimmermann, 2001). The presence of ectonucleotidase was shown experimentally both in the endolymphatic (Vlajkovic et al., 1998a) and perilymphatic compartments (Vlajkovic et al., 1998b; Vlajkovic et al., 1996). Of six members of the E-NTPDase family, NTPDase 1, NTPDase 2, and NTPDase 3 are likely the key elements for termination of purinergic signaling in the cochlea (Vlajkovic et al., 1999; Vlajkovic et al., 2006). NTPDase 1 degrades both nucleoside triphosphates and diphosphates. In contrast, NTPDase 2 has preference for nucleoside triphosphate. NTPDase 3 is intermediate in terms of the nucleotide hydrolysis ratio (ATPase: ADPase activity) (Smith and Kirley, 1998). Immunohistochemical staining revealed the detailed location of NTPDase 1, 2, and 3 in the murine cochlea (Vlajkovic et al., 2002a; Vlajkovic et al., 2002b; Vlajkovic et al., 2006). The distribution of E-NTPDases in the cochlear vasculature, spiral ligament, spiral ganglion neuron, cuticular plate of inner and outer hair cells, and afferent dendritic synapse indicates that they involve a variety of cochlear physiologic processes in which purinergic P2X and P2Y receptors are expressed.

Activation by noise and/or stress

All three components of the mechanism of purinergic signaling (agonist, receptor, termination) in the cochlea can be activated by acoustic-overstimulation. The increase of agonist with noise was described above. Chronic noise exposure also upregulates P2X_2 mRNA in cochlear hair cells, spiral ganglion (Wang et al., 2003), adenosine receptor in the cochlear tissues (Ramkumar et al., 2004), NTPDase 1 and 2 mRNA in the cochlea (Vlajkovic et al., 2004), and NTPDase 3 immunoreactivity at sites associated with auditory neurotransmission (Vlajkovic et al., 2006). NTPDase 1 and 2 was upregulated in the stria vascularis and NTPDase 2 in intraganglionic spiral bundle, but NTPDase 1 was down-regulated in spiral ganglion neurons (Vlajkovic et al., 2004).

P1 (A) adenosine receptors

There is a family of four adenosine receptors (P1 receptors or A receptors) which have been cloned and characterized from several mammalian species. These include A_1 , $\text{A}_{2\text{A}}$, $\text{A}_{2\text{B}}$, and A_3 (Fredholm et al., 2001). A_1 and A_3 receptors couple to $\text{G}_{i/o}$ -dependent pathway and inhibit adenylyl cyclase, and $\text{A}_{2\text{A}}$ and $\text{A}_{2\text{B}}$ receptors preferentially interact with the members of the G_s family of G proteins. A_1 receptors have been identified in rat cochlear tissues using radioligand binding studies and RT-PCR (Ramkumar et al., 1994). Further, the expression level of A_1 and A_3 receptors was found to be higher in the well-vascularized cochlear lateral wall and organ of Corti than in the eighth nerve of chinchilla cochlea (Ford et al., 1997). Recently, mRNA transcripts for all four subtypes of adenosine receptors were detected in rat cochlear tissue, and adenosine receptors were found to be differentially expressed in the sensory hair cells and supporting cells of the organ of Corti, spiral ganglion neurons, and cochlear vasculature (Vlajkovic et al., 2007).

Several studies have indicated the presence in cochlear tissues of adenosine receptors that are thought to be activated through generation of reactive oxygen species (ROS) in response to various stresses to the inner ear. Noise exposure induced A₁ receptor expression, which might be mediated, in part, through generation of ROS and activation of nuclear factor (NF)-κB (Ramkumar et al., 2004). In particular, there is evidence that cochlear blood flow can be modulated by adenosine receptors. The application of adenosine to the perilymph (scala tympani) produced a dose-dependent increase in cochlear blood flow, as measured by a laser Doppler blood flow probe on the bone overlying the cochlear lateral wall (Thorne and Nuttall, 1987). Further, the agonist adenosine is released by ROS-generating systems, and attenuates the deleterious cellular consequences of ROS through A₁ receptor activation (Almeida et al., 2003; Ramkumar et al., 2004). The P₁ signal pathway therefore is likely a key player in protection of cochlear function from stresses such as cochlear hypoperfusion and ischemia, noise exposure, aging, and ototoxicity that are mediated by generation of ROS (Ohinata et al., 2000; Ohlemiller et al., 1999; Seidman et al., 1999; Yamashita et al., 2004).

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
E-NPP	ectonucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase
MARCKS	myristoylated alanine-rich C-kinase substrate
OSC	outer sulcus cells
I_{sc}	inner sulcus cells
PIP₂	phosphatidylinositol-4,5-bisphosphate
IP₃	inositol 1,4,5-trisphosphate

PKC	protein kinase C
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid
RM	Reissner's membrane
ROS	reactive oxygen species
ScM	scala media
ScT	scala tympani
ScV	scala vestibuli
SMC	strial marginal cells
SV	stria vascularis
UTP	uridine triphosphate
VDC	vestibular dark cells
VTC	vestibular transitional cells

Fig. 1.

Distribution and function of purinergic receptors in the inner ear.

(A) A schematic drawing of the cochlea. The membranous labyrinth of the cochlea, comprised of scala media (ScM) filled with endolymph (high K^+ , low Na^+), and scala vestibuli (ScV) and tympani (ScT) with perilymph (high Na^+ , low K^+). Sound energy delivered to the oval window generates a traveling wave along the cochlear duct. Auditory transduction is initiated by activation of the mechanoelectric transduction channels in the stereocilia of the inner hair cells through which endolymphatic K^+ enters. Depolarization of inner hair cells leads to Ca^{2+} influx via voltage-gated Ca^{2+} channels at the basolateral membrane and subsequent release of neurotransmitter to the afferent dendrites of the spiral ganglion. Three rows of outer hair cells also utilize K^+ in response to sound and provide an amplification mechanism to the inner hair cells. K^+ used by the inner and outer hair cells recycles to ScM by diffusion through perilymph to the lateral wall and has been proposed to also move through medial (inner sulcus cells; ISC) and/or lateral gap junction pathways (dotted arrows; reviewed in Wangemann, 2006). The stria marginal cells (SMC) in the stria vascularis (SV) secrete K^+ , which is the substrate for auditory transduction. The outer sulcus cells (OSC) provide pathways for parasensory cationic absorption. Na^+ is also extruded from the endolymph to perilymph via epithelial Na^+ channels in Reissner's membrane (RM). $P2X_2$ purinergic receptors are expressed in many of the epithelial cells bordering endolymph (red cells) except stria vascularis where apical $P2Y_4$ and basolateral $P2Y_2$ purinergic receptors are expressed (blue cells). $P2X_2$ expressed at the

stereocilia of the hair cells, afferent dendrites of spiral ganglion, and body of spiral ganglion is not shown.

(B) Schematic diagram of modulation of K^+ secretion by $P2Y_4$ receptors in SMC and vestibular dark cells (VDC). The pathways are described in the text. Membrane transporters are labeled in red font; $P2Y$ receptors in blue font. G, heterotrimeric G protein; PLC, phospholipase C; PIP_2 , phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; IP_3 , inositol 1,4,5-trisphosphate; PKC, phosphokinase C; CaM, calmodulin; MKS, myristoylated alanine-rich C-kinase substrate (MARCKS).

(C) A schematic drawing of the ampulla of the semicircular canal. The vestibular system consists of three semicircular canals, saccule, and utricle. The hair cells in the ampulla of the semicircular canal detect angular acceleration, and the ones in the saccule and utricle detect linear acceleration. The VDC and vestibular transitional cells (VTC) have homology with SMC and OSC, respectively, in terms of the ion transport mechanisms and purinergic signaling. $P2X_2$ is expressed in VTC and the supporting cells surrounding the hair cells (red cells). In VDC, $P2Y_4$ is expressed at the apical membrane and $P2Y_2$ is expressed at the basolateral membrane (blue cells).

(D) Ion transport model for OSC and VTC illustrating both ATP-sensitive ($P2X_2$ receptor) and ATP-insensitive nonselective cation (NSC) channels in the apical membrane mediating absorption of Na^+ , K^+ and Ca^{2+} from endolymph. Na^+ entering the cell through these channels is removed across the basolateral membrane by the Na^+ -pump. K^+ entering the cell via these apical channels and via the Na^+ -pump diffuse out of the cell through the basolateral K^+ channel (**A** and **C** adapted from (Wangemann, 2002).)