ATP-mediated potassium recycling in the cochlear supporting cells

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Abstract Gap junction-mediated K^+ recycling in the cochlear supporting cell has been proposed to play a critical role in hearing. However, how potassium ions enter into the supporting cells to recycle K^+ remains undetermined. In this paper, we report that ATP can mediate K^+ sinking to recycle K^+ in the cochlear supporting cells. We found that micromolar or submicromolar levels of ATP could evoke a K^+ -dependent inward current in the cochlear supporting cells. At negative membrane potentials and the resting membrane potential of -80 mV, the amplitude of the ATP-evoked inward current demonstrated a linear relationship to the extracellular concentration of K^+ , increasing as the extracellular concentration of K^+ increased. The inward current also increased as the concentration of ATP was increased. In the absence of ATP, there was no evoked inward current for extracellular K^+ challenge in the cochlear supporting cells. The ATP-evoked inward current could be inhibited by ionotropic purinergic (P2X) receptor antagonists. Application of pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 50 μ M) or pre-incubation with an irreversible P2X7 antagonist oxidized ATP (oATP, 0.1 mM) completely abolished the ATP-evoked inward current at the negative membrane potential. ATP also evoked an inward current at cell depolarization, which could be inhibited by intracellular Cs^+ and eliminated by positive holding potentials. Our data indicate that ATP can activate P2X receptors

to recycle K^+ in the cochlear supporting cells at the resting membrane potential under normal physiological and pathological conditions. This ATP-mediated K^+ recycling may play an important role in the maintenance of cochlear ionic homeostasis.

Keywords ATP · Potassium · P2x receptor · Purinergic signaling · Gap junction · Connexin · Cochlea · Deafness

Introduction

Supporting cells in the cochlea provide physical support and nutrition to hair cells and also play an important role in the maintenance of cochlear ionic homeostasis [1, 2]. It has been hypothesized that supporting cells in the cochlea like glia cells in the brain absorb or sink K^+ ions, which hair cells release during mechano-electrical transduction, and transport them back to the endolymph via intracellular gap junctional communication [3–9]. However, the detailed mechanism by which potassium ions enter into the cochlear supporting cells to recycle K^+ remains unclear.

ATP is an important extracellular signaling molecule. In the cochlea, it has been reported that ATP can evoke inward currents and raise the intracellular Ca^{++} concentration in the outer and inner hair cells, thereby modifying sound transduction and neurotransmission [10–13]. ATP can activate purinergic (P2) receptors to produce inward cationic currents [14, 15]. P2 receptors have two subgroups: ATP-gated ionotropic (P2X) receptors and G-protein-coupled metabotropic (P2Y) receptors. Both P2X and P2Y receptors are expressed in the cochlea, including supporting cells [16–23]. Recently, we have demonstrated that gap junctional hemichannels in the cochlear supporting cells can release ATP [24], which can activate P2x receptors in the outer hair cells

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(OHCs) to induce Ca^{++} influx and modulate OHC electromotility [24, 25]. P2X receptors have a cationic permeability, permeable to K^+ ions [15]. In this study, the effect of ATP on K^+ recycling in the cochlear supporting cells was investigated. We found that the micromolar and submicromolar levels of ATP can induce a significant K^+ -dependent inward current in the cochlear supporting cells at the resting membrane potential. Our new findings indicate that ATP can mediate K^+ sinking and recycling in the cochlear supporting cells and plays an important role in cochlear ionic homeostasis.

Materials and methods

Animal preparation and cochlear supporting cell isolation

Cochlear supporting cells were freshly isolated from adult guinea pigs (250–400 g, $n=43$) as previously described [8, 26]. Briefly, the temporal bones were removed after decapitation.

The otic capsule was isolated and dissected in normal extracellular solution (in mM: 130 NaCl, 5 KCl, 1.47 MgCl_2 , 2 CaCl_2 , 25 dextrose, and 10 HEPES; 300 mOsm, pH 7.2) to reveal the organ of Corti. The sensory epithelium was microdissected by a sharpened needle. The isolated sensory epithelium was dissociated by trypsin (1 mg/ml) for 5–10 min. The dissociated cells were then transferred to a dish for recording. All experimental procedures were conducted at room temperature (23°C) in accordance with the policies of the University of Kentucky Animal Care & Use Committee.

Patch-clamp recording and data processing

Single dissociated cochlear supporting cell was selected and recorded under the whole cell configuration (Fig. 1a) using an Axopatch 200B patch clamp amplifier (Molecular Devices, CA, USA). Patch pipettes were filled with an intracellular solution that contained (in mM) 140 KCl, 5 EGTA, 2 MgCl_2 , and 10 HEPES, pH 7.2, with initial resistance of

Fig. 1 Cochlear supporting cells and ATP-evoked inward current. **a** Micrographs of isolated single cochlear supporting cell and patch clamp recording. *DC* Deiters' cell, *HC* Hensen cell, *PC* pillar cell, *CC* Claudius cell. Scale bars, 10 μm. **b**, **c** Current traces evoked by ATP in a Deiters' cell. The cell membrane potential was clamped at -80 mV (**b**) and 0 mV (**c**). The horizontal bars represent the application of 36 μM

ATP. **d**, **e** Evoked currents in a Deiters' cell for voltage step stimulation. Red and blue colors represent current responses to the positive and negative voltage step stimuli, respectively. The current-voltage (*I-V*) relations were plotted by average values of the steady-state currents in last 20 ms of the voltage step stimulation. Pink color represents the *I-V* curve of the subtracted inward current evoked by ATP

2.5–3.5 M Ω in bath solution. Data were collected by jClamp software (SciSoft, New Haven, CT, USA) [7–9, 25]. The signal was filtered by a four-pole low-pass Bessel filter with a cutoff frequency of 2 kHz and digitized utilizing a Digidata 1322A (Molecular Devices, CA, USA).

Data were analyzed with jClamp and plotted by SigmaPlot software (SPSS Inc. Chicago) for presentation. Membrane potential (V_m) was corrected for pipette series resistance (R_s). Error bars represent SE.

Potassium challenge and chemical perfusion

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA). A Y-tube perfusion system was used for the application of ATP and chemicals [25]. The potassium challenge was achieved by perfusion with high K⁺ extracellular solutions, which were prepared by replacement of NaCl with KCl in the normal extracellular solution. The solution osmolarity was kept constant at 300 mOsm.

Results

ATP-evoked inward current in the cochlear supporting cells

The organ of Corti of guinea pigs contains four types of supporting cells, i.e., Deiters' cell, pillar cells, Hensen cells,

and Claudius cells, with their own morphological characteristics (Fig. 1a; also see [26]). ATP could evoke the inward currents in all tested cochlear supporting cells ($n > 100$, Figs. 1 and 2). The evoked inward currents in the cochlear supporting cells show two phases: a large, quick phase followed by a delayed, developing phase (Fig. 1b). This is a characteristic of P2X receptor activity [15]. The evoked inward current was large at the resting membrane potential of -80 mV and became invisible at the membrane potential of 0 mV (Fig. 1b, c). The inward current was also visible at cell depolarization, showing a bell shape for the evoked I - V curves (Figs. 1e and 2f). The evoked inward current increased when the cell was hyperpolarized and depolarized and was large at the negative membrane potential (Figs. 1d, e and 2c–f).

Potassium dependence of ATP-evoked inward current in the cochlear supporting cells

The evoked inward current depended on extracellular K⁺ (Fig. 3). As the extracellular concentration of K⁺ was increased, the ATP-evoked inward current increased (Fig. 3a). At the resting membrane potential of -80 mV, the amplitudes of the ATP-evoked inward currents at 5, 10, and 20 mM extracellular K⁺ concentrations were -0.52 ± 0.13 ($n=8$), -0.96 ± 0.23 ($n=7$), and -1.89 ± 0.24 ($n=19$) nA, respectively. The regression analysis shows good linear

Fig. 2 ATP-evoked inward current in the cochlear supporting cells. **a**, **b** Current responses of a Hensen cell to voltage step stimulation. Red and blue colors represent the current responses to positive and negative voltage step stimuli, respectively. The I - V curve was plotted by average values of the steady-state currents in the last 20 ms of

voltage step stimulation. **c**, **d** Current trace and I - V curve under $36 \mu\text{M}$ ATP perfusion. **e**, **f** ATP-evoked currents and I - V curve. The current response was obtained by subtraction of the control current response from that under ATP application. The inward current increased when the cell was depolarized and hyperpolarized. R_s , $6.9 \text{ M}\Omega$

Fig. 3 K^+ dependence in the ATP-evoked inward currents in the cochlear supporting cells. **a** I - V relations evoked by ATP (36 μM) at different extracellular concentrations of K^+ . The evoked inward currents at negative membrane potentials increased as the extracellular K^+ concentration was increased. **b** The increase in the amplitude of the inward current at negative membrane potentials has linear relationships to the extracellular concentration of K^+ ($[\text{K}^+]_o$). Solid lines represent data fitted by a linear function ($y=ax+b$; $a=-0.036$, -0.091 , and -0.167 nA/mM for $V_m=-40$, -80 , and -120 mV, respectively). r is the coefficient of linear regression. Error bars represent SE

relationships ($r>0.99$) between the amplitudes of inward currents and the extracellular concentrations of K^+ at negative membrane potentials (Fig. 3b). The slope was 0.036, 0.091, and 0.167 nA/mM at the holding potentials of -40 , -80 , and -120 mV, respectively, and increased as the cell became more hyperpolarized.

ATP dependence of the inward current in the cochlear supporting cells

Figure 4 shows the evoked inward current in the cochlear supporting cell by application of micromolar and submicromolar levels of ATP. The inward current could be evoked by nanomolar ATP at the physiological level (Fig. 4b) and increased as the concentration of ATP was increased (Fig. 4a). However, in the absence of ATP, extracellular K^+ challenge could not evoke an apparent inward current in the cochlear supporting cells (Fig. 5). There was no visible inward current evoked by increasing the extracellular concentration of K^+ from 5 to 20 mM in

Fig. 4 Physiological level of ATP evoked inward currents in the cochlear supporting cells. **a** Inward currents in a Hensen cell evoked by micromolar and submicromolar levels of ATP. **b** Nanomolar ATP evoked inward current in a Hensen cell

Fig. 5 Absence of ATP cannot evoke an inward current for high extracellular K^+ challenge in the cochlear supporting cells. **a** I - V relations of a Hensen cell at normal (5 mM) and high (20 mM) extracellular concentrations of K^+ in the absence of ATP. *Inset* Current traces to voltage step stimuli at 5 and 20 mM extracellular concentrations of K^+ . R_s , 5.97 M Ω . **b** Response for high (20 mM) extracellular K^+ challenge in the absence of ATP. The curve is obtained from the subtraction of the current response at 5 mM $[K^+]_o$ from that at 20 mM $[K^+]_o$ and averaged. *Inset* Current traces obtained after subtraction from the *inset* in **a**. There is no inward current visible

the absence of ATP in all ten cells tested (Fig. 5b). Actually, a small outward current is visible at cell depolarization since high extracellular K^+ challenge can cause cell depolarizing. Therefore, ATP is required for high K^+ -induced inward currents in the cochlear supporting cells.

Blockage of inward current by P2X receptor antagonists

The ATP-induced inward current could be blocked by P2X receptor antagonists (Figs. 6 and 7 and Electronic supple-

mentary material (ESM) Fig. S1). Figure 6 shows that pre-application of pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 50 μ M) inhibited the ATP-evoked current in the cochlear supporting cells. PPADS completely abolished the ATP-evoked inward current at the negative

Fig. 6 Inhibition of the ATP-evoked inward current in the cochlear supporting cells by a P2X receptor blocker PPADS. **a** Current trace in a Hensen cell evoked by ATP before and after perfusion of PPADS. Horizontal bars represent ATP (36 μ M) and PPADS (50 μ M) perfusion. Pre-application of PPADS inhibited ATP to evoke inward current. The cell was held at -80 mV. **b** Current traces of a Deiters' cell for ATP application at voltage step stimulation with and without PPADS (50 μ M) treatment. The current traces were subtracted by control current responses. See ESM Fig. S1 for other current traces and I - V curves. **c** I - V relations for applications of 36 μ M ATP before (control) and after treatment of PPADS (50 μ M). The data were averaged from the responses of the same supporting cells to ATP (36 μ M) before and after application of PPADS (50 μ M). PPADS completely inhibited the ATP-evoked inward current at the negative membrane potential

Fig. 7 Blockage of the ATP-evoked inward current at negative membrane potentials by a P2X7 antagonist, oxidized ATP (oATP). **a, b** ATP-evoked inward current in a pillar cell. Horizontal bar in **a** represents the application of 36 μ M ATP. Membrane potential was held at -80 mV. R_s , 5.7 M Ω . **c, d** Inhibition of the ATP inward currents at negative membrane potentials by pretreatment of oATP. The cells were pre-incubated with 0.1 mM oATP for 45 min. Horizontal bar in **c** represents the application of 36 μ M ATP. The cell membrane potential was held at -80 mV. Inset in **d** shows ATP-evoked current traces after pre-incubation with oATP. Red and blue colors represent the current responses to positive and negative voltage step stimuli, respectively

membrane potential (Fig. 6b, c and ESM Fig. S1). The ATP-evoked inward current at the positive membrane potential was reduced but still visible (Fig. 6b, c and Fig. S1), indicating that other mechanisms may also contribute to the production of the inward current at cell depolarization.

Oxidized ATP (oATP), which can irreversibly block the P2X7 receptor, also completely blocked the ATP-evoked inward current at the negative membrane potential in the cochlear supporting cells (Fig. 7, four experiments). After the pre-incubation of 0.1 mM of oATP for 45 min, the ATP-evoked inward current at the negative membrane potential was completely blocked (Fig. 7c, d). Similarly, the ATP-evoked inward current retained at cell depolarization (Fig. 7d).

Inhibition of the ATP-evoked inward current at cell depolarization by intracellular Cs^+ and positive holding potentials

Positive holding potential and intracellular Cs^+ could eliminate the ATP-evoked inward current at cell depolar-

ization, but little affected the inward current at negative membrane potentials (Figs. 8 and 9 and ESM Fig. S2). Figure 8 shows that holding potential of $+20$ mV eliminated the ATP-evoked inward current at cell depolarization. However, the evoked inward current at the negative membrane potential was not affected (Fig. 8 and ESM Fig. S2).

Figure 9 shows the ATP-evoked current in the cochlear supporting cells recorded by Cs^+ patch pipette. Cs^+ can block potassium channels. ATP-evoked inward current at the negative membrane potential was still visible and large in the Cs^+ pipette recording (Fig. 9 and ESM Fig. S3). However, the evoked inward current at cell depolarization disappeared (Fig. 9b), revealing the rectification of the P2X receptor conductance.

Discussion

In this study, we found that ATP evoked a K^+ -dependent inward current in the cochlear supporting cells (Figs. 1, 2,

Fig. 8 Elimination of ATP-evoked inward currents at cell depolarization by positive holding potential inactivation. **a** Current traces of a Claudius cell for voltage step stimulation from -150 to +70 mV at holding potentials of -80 and +20 mV. Red and blue colors represent the ATP-evoked current responses to positive and negative voltage step stimuli, respectively. The current traces were submitted by control responses (see ESM Fig. S2). The cell was perfused with 36 μ M ATP. **b** I - V relations evoked by ATP at the holding potentials of -80 and +20 mV in the cochlear supporting cells. The curves were averaged from the responses of the same cells holding at -80 and +20 mV to the 20 mM $[K^+]_o$ challenge in the presence of 36 μ M ATP. Positive holding potential abolished the ATP-evoked inward currents at cell depolarization, but little affected the inward currents at negative membrane potentials

3, and 4). The evoked inward current increased as the extracellular concentration of K^+ was increased (Fig. 3). In the absence of ATP, there was no evoked inward current for extracellular K^+ challenge in the cochlear supporting cells (Fig. 5). These data indicate that ATP can induce K^+ sinking to recycle K^+ in the cochlear supporting cells.

ATP physiologically exists in the cochlear endolymph and perilymph. Under normal physiological conditions, the cochlear endolymph and perilymph contain nanomolar amounts of extracellular ATP [27]. It has been found that cochlear ATP is mainly released from cochlear supporting

cells via gap junction hemichannels [24]. In the local area near the cell surface, the ATP concentration would be high and can reach micromolar levels [24, 28]. In this study, we found that the application of submicromolar and nanomolar ATP could evoke inward currents in the cochlear supporting cells (Fig. 4). Moreover, our records show that the evoked inward current increased as the cell became hyperpolarized (Figs. 1, 2, 3, and 4), demonstrating linear relationships to the extracellular concentration of K^+ at negative membrane potentials (Fig. 4). The slope increased as cells were hyperpolarized (Fig. 4). Hence, this ATP-mediated K^+ sinking may be able to function under normal physiological conditions and play an important role in the cochlea for K^+ recycling.

Fig. 9 Inhibition of the ATP-evoked inward current at positive membrane potentials by intracellular Cs^+ . The cell was recorded on the whole cell configuration using a Cs pipette, which was filled with the Cs-based intracellular solution that 140 mM K^+ was replaced by 140 mM Cs^+ . **a** Inward current evoked by ATP (36 μ M) with Cs-pipette at a Claudius cell. The cell was held at -40 mV. **b** ATP-evoked I - V relation in the cochlear supporting cells with the Cs pipette. Inset: Subtracted ATP-evoked current trace. Red and blue colors represent the responses to positive and negative voltage stimuli, respectively. See ESM Fig. S3 for other current traces and I - V curves. The ATP-evoked current at cell depolarization was reversed and became outward

This ATP-evoked inward current was inhibited by P2X receptor antagonists (Figs. 6 and 7 and ESM Fig. S1), indicating that ATP sinks K^+ through the activation of the P2X receptors. Multiple expression of P2X isoforms have been identified in the cochlea, including supporting cells [18, 20, 21, 23, 29, 30]. P2X2, P2X4, and P2X7 are the predominant isoforms. These P2X isoforms can form homomeric and heteromeric channels to influx cations when ATP binds to the binding site [15, 31]. The recorded inward current also shows slow desensitization (Figs. 1b, 6a, 7a, and 9a), which is a known characteristic of P2X receptor activity [15]. PPADS and oATP completely inhibited the ATP-evoked inward current in the cochlear supporting cells at negative membrane potentials, but had little effect on the evoked inward current at cell depolarization (Figs. 6 and 7c, d and ESM Fig. S1). This is also consistent with previous reports that inward currents pass more readily than outward currents through the P2X receptors, a characteristic referred to as inward rectification [32, 33].

The ATP-evoked inward current was also visible at cell depolarization (Figs. 1, 2, 3, 4, 6, 7, 8, and 9) and insensitive to PPADS and oATP applications (Figs. 6 and 7 and ESM Fig. S1), implying that other mechanisms also exist beyond the P2X receptor activity. We found that the inward current at cell depolarization was eliminated by deactivation of positive holding potentials (Fig. 8) and could be inhibited by intracellular Cs^+ (Fig. 9), suggesting that other K^+ -dependent channels, such as Ca^{++} -activated K (K_{Ca}) channels [34], may be involved. Currently, the detailed mechanism underlying this evoked inward current at cell depolarization remains unclear. Further studies are required.

The evoked inward currents increased as the ATP concentration was increased (Fig. 4). We have reported that cochlear gap junctional hemichannels can release ATP and inositol 1,4,5-trisphosphate (IP_3) [24, 35]. Such release increased under mechanical stimulation. Moreover, we have recently reported that a new gap junction gene family Pannexin is extensively expressed in the inner ear [36]. Pannexins mainly assemble functional hemichannels, which can also release ATP [37]. It has also been reported that noise can increase the ATP level in the cochlea [38]. Loud sound stimulation increases the potassium level around the hair cells [39]. Such increase in ATP release can in turn enhance K^+ sinking in the cochlear supporting cells (Figs. 1, 2, 3, and 4), conferring protection from K^+ toxicity. Recently, we have found that current or voltage changes in Deiters' cells can modify outer hair cell electromotility [40], which is an active cochlear amplifier and can increase auditory sensitivity and frequency selectivity in mammals. Thus, this ATP-mediated K^+ sinking mechanism may play an important role in protecting the cochlea from noise damage and also have an implication in hearing regulation.

Cochlear supporting cells are well coupled by gap junctions [5, 41–43]. Dysfunction of gap junctions can induce a high incidence of hearing loss [1]. For a long time, researchers have hypothesized that the inner ear gap junctions mediate K^+ transport back to the endolymph [2–5, 7–9]. In this study, we found that ATP can induce K^+ sinking in the cochlear supporting cells. This is the first step for K^+ transport and provides direct evidence for K^+ recycling in the cochlea through supporting cells.

ATP can also activate P2X receptors to mediate cation absorption in the hair cells and outer sulcus cells in the cochlear lateral wall [44, 45] and other signaling events in the cochlea [46]. In previous studies, we have reported that gap junctions and hemichannels in the cochlea can release ATP and IP_3 to mediate or control nutrient and energy supplies in the cochlea [24, 26, 35, 47, 48]. In this study, we found that ATP can activate P2X receptors to mediate K^+ sinking and recycling in the cochlear supporting cells. Therefore, ATP not only mediates the cochlear nutrition supplies but also plays an important role in the maintenance of the cochlear ionic homeostasis.

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