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Adenosine Triphosphate (ATP) Inhibits Voltage-Sensitive Potassium Currents in Isolated Hensen's Cells and Nifedipine Protects Against Noise-Induced Hearing Loss in Guinea Pigs

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Background: There is increasing evidence that adenosine triphosphate (ATP), a well-known neurotransmitter and neuro-modulator in the central nervous system, plays an important role as an extracellular chemical messenger in the cochlea.



Material/Methods: Using a whole-cell recording technique, we studied the effects of ATP on isolated Hensen's cells, which are supporting cells in the cochlea, to determine if they are involved in the transduction of ions with hair cells.

Results: ATP (0.1–10 μM) reduced the potassium current (I_{K}^+) in the majority of the recorded Hensen's cells (21 out of 25 cells). An inward current was also induced by high concentrations of ATP (100 μM to 10 mM), which was reversibly blocked by 100 μM suramin (a purinergic antagonist) and blocked by nifedipine (an L-type calcium channel blocker). After the cochleas were perfused with artificial perilymph solutions containing nifedipine and exposed to noise, the amplitude increase in the compound action potential (CAP) threshold and the reduction in cochlear microphonics was lower than when they were exposed to noise alone.

Conclusions: Our results suggest that ATP can block I_{K}^+ channels at a low concentration and induce an inward Ca^{2+} current at high concentrations, which is reversed by purinergic receptors. Nifedipine may have a partially protective effect on noise-induced hearing loss (NIHL).

MeSH Keywords: Adenosine Triphosphate • Hearing Loss, Noise-Induced • Kv Channel-Interacting Proteins • Nifedipine • Organizers, Embryonic

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Background

The organ of Corti includes 2 types of cells: sensory cells (hair cells) and supporting cells [1]. The electromotive outer hair cells are believed to actively amplify the signal from the cochlea in response to low sound stimuli [2]. To maintain this highly sensitized function of hair cells, the surrounding supporting cells provide a suitable electrical and micromechanical environment for the hair cells to maintain homeostasis of the organ of Corti. Cell-to-cell connections through gap junctions between outer hair cells and supporting cells or between supporting cells and supporting cells were identified in different types of supporting cells (e.g., Hensen's cells and Deiter's cells), which were believed to play important roles in maintaining the normal physiological environment and functions of hair cells [3,4]. Gap junctions between the supporting cells and hair cells maintain the stable concentration difference of K^+ between the endolymph and the perilymph by forming a K^+ ion recycling pathway from the sensory hair cells to the endolymph [5]. ATP, existing in the endolymph and perilymph of the cochlea, is believed to be an important modulator of this ion transport mechanism [6].

Noise exposure elevates ATP levels in this cochlear compartment and changes the K^+ conductance through P_{2X} receptors by reducing the endocochlear potential (EP) [7]. In guinea pig cochlear fluids, extracellular ATP concentrations were elevated after a short 15-min noise exposure [8]. The observation that the efflux of K^+ from the scala media is complemented by a P_{2Y} receptor, a G protein-coupled ATP sensitive pathway, supports this hypothesis. This purinergic signaling mechanism is likely to provide the basis for a reactive homeostatic regulatory mechanism that limits cochlear sensitivity under stressor conditions. Loss function of the ATP-gated $P2X2$ receptor (ligand-gated ion channel, purinergic receptor) which is expressed in sensory and supporting cells of the cochlea, may cause NIHL [9]. It is reasonable to speculate that ATP may influence the K^+ currents of Hensen's cells, although little is known about this suppressive effect.

Hearing loss due to noise exposure is a common sensorineural hearing impairment in industrialized countries [10]. White noise exposure at 120 dB SPL (sound pressure level) for 1 h induced a CAP (compound action potential) threshold shift and a CM (cochlear microphonic) potential amplitude shift [11]. Ionized calcium (Ca^{2+}), a second messenger, plays a critical role in cellular function, including cell proliferation, differentiation, and apoptosis [12]. However, a sustained increase in intracellular Ca^{2+} levels is toxic to cells, leading to cell death [13]. Several factors may contribute to the elevated Ca^{2+} concentration of the cochlea, such as noise exposure and mechanical overstimulation, and acoustic trauma can increase the intracellular Ca^{2+} , thus increasing the ototoxicity and causing narrowed

chamber and increased permeability of the vessel, resulting in ischemia-hypoxia and energy metabolism disorders (e.g., ATP depletion) of the cochlea [14,15]. Therefore, it is reasonable to speculate that NIHL is related to the abnormal calcium homeostasis of the cochlea.

The regulators of calcium homeostasis, including a calcium channel blocker and an indirect stimulator of the Ca^{2+} pump, are involved in the regulation of intracellular Ca^{2+} . According to the effects of Ca^{2+} on cells and blood vessels, a typical calcium channel blocker, nifedipine, which can reduce Ca^{2+} influx and dilate blood vessels to relieve spasm and contraction induced by noise, was chosen to be studied in this experiment. The purposes of the present study were: 1) to research the influence of ATP on potassium current in isolated adult Hensen's cells by use of the whole-cell recording technique; 2) to determine the effect of nifedipine (an L-type calcium channel blocker) on the cochlea of guinea pigs by examining EP; and 3) to identify whether it has a preventive effect on NIHL by reducing the concentration of intracellular Ca^{2+} .

Material and Methods

Ethics statement

The entire experiment is done in the Academy of Military Medical Sciences (AMMS). Animal breeding and care and all experiments were performed in adherence to AMMS Experimental Animal Center guidelines and were approved by the Experimental Animal Ethics Committee of AMMS.

Isolation of Hensen's cells from the cochlea

Pigmented guinea pigs (250–300 g) with a positive Preyer's reflex were sacrificed using an overdose of sodium pentobarbital (50 mg/kg). The cochlea was then harvested and the bony shell of the cochlea was gently removed. The organ of Corti was dissected and incubated in Hanks' balanced salt solution (HBSS). A cell suspension was obtained by digestion for 30 min with trypsin (0.25 mg/ml) in nominal Ca^{2+} -free HBSS. The cell suspension was washed with HBSS and transferred into a perfusion chamber. Isolated Hensen's cells were identified by their characteristic lipid inclusions and cultured on poly-L-lysine-coated coverslips (Figure 1) [16].

Preparation of solutions

For the patch clamp experiments, the extracellular solution contained the following (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 2.6, NaH_2PO_4 1.25, and glucose 10. The pipette filling solution for recording K^+ currents contained the following (in mM): KCl 140, HEPES 10, $MgCl_2$ 1, and EGTA 10.

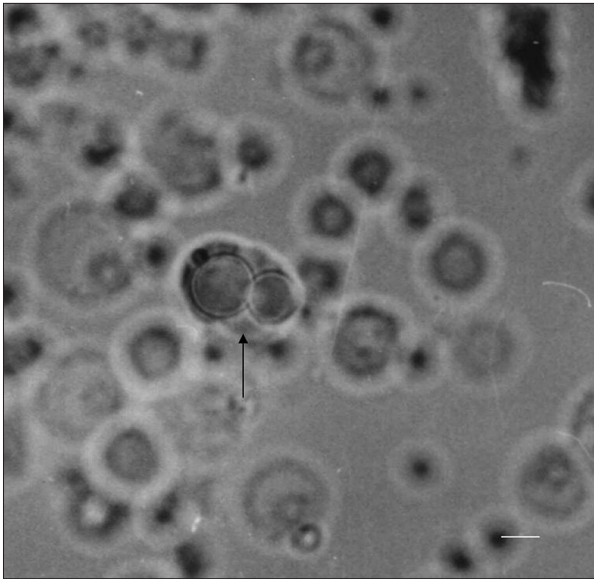


Figure 1. A typical Hensen's cell isolated from the cochlea of an adult guinea pig (scale bar: 10 μm).

All solutions were buffered to pH 7.3 with NaOH and the osmolarity was adjusted to 300 mOsm with glucose. ATP, TEA (tetraethylammonium), suramin, and nifedipine were purchased from Sigma. ATP and suramin were dissolved in the extracellular solution. Nifedipine was dissolved in dimethyl sulfoxide and then added to the extracellular solution.

Electrophysiological recordings

Pipettes were pulled using a 2-stage puller (Sachs-Flamming PC-84; Sutter Instruments, USA) to a resistance of 2–5 M Ω when filled with the pipette solution. Drugs were delivered to the cell using a micro-manifold consisting of 3 microtubules with a 100 μm diameter. The drugs were applied directly to single cells. The opening of the microtubule was placed approximately 1 mm from the recorded cell and the perfusion pressure of N₂ (controlled using a BH-2 pressure injector made by Medical Systems Corp.) was adjusted to achieve rapid drug application while avoiding any mechanical disturbance to the recording. One of the microtubules was filled with the extracellular solution as a control and the others were filled with different concentrations of ATP as the test groups. Whole-cell recordings (Axopatch-1D; Axon Instruments Inc., USA) were performed at room temperature (21–25°C). Data acquisition and analysis were accomplished using pCLAMP 7.0 software (Axon instruments). The dose-response curve was fitted using Sigma Plot 2000 software (SPSS Inc., USA) with the logistic equation. Origin software 7.0 (OriginLab Inc., USA) was used for the I-V graphic display and SAS 6.12 software (SAS Inc., USA) was used to conduct the 2-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant.

Artificial perfusion with nifedipine

After the guinea pigs were anesthetized, a small hole was drilled in the wall of the scala tympani and the scala vestibule in the basal turn of the cochlea. The prepared solution was perfused through the fenestra in the scala tympani and released through an outlet in the scala vestibule at a speed of 3 $\mu\text{l}/\text{min}$ for 2 h. The right ear of the animals was exposed to white noise at 120 dB SPL for the noise exposure. Clicks at 10–90 dB SPL were used as acoustic stimuli. A recording electrode was placed on the round window niche. The reference electrode was placed in the neck muscle. Forty healthy hybrid guinea pigs were randomly divided into 4 groups. The perilymphatic spaces of the guinea pig cochleas were perfused with artificial perilymph solutions containing no or 0.5 $\mu\text{mol}/\text{L}$ nifedipine with or without noise exposure for 2 h. CAP and CM were recorded from the round windows of the guinea pigs before and 120 min after perfusion.

Results

ATP depresses the outward K⁺ currents of Hensen's cells

Voltage-dependent potassium currents (I_{K}^+) of Hensen's cells were elicited using voltage steps from -90 to 60 mV (10 mV steps) (Figure 2A). A typical I/V curve of the I_{K}^+ recorded from Hensen's cells is shown in Figure 2B. The outward currents could be totally blocked using Cs⁺ (140 mM) in the pipette and TEA (40 mM) in the bath solution, indicating that these currents are carried by K⁺ (Figure 2C).

I_{K}^+ was significantly depressed by the direct application of ATP to the cell body and partially recovered at 2–3 min after ATP was washed out (Figure 3A). The reduction of I_{K}^+ was increased in an ATP-dependent manner from 0.1–10 μM . The mean [\pm standard deviation (SD)] suppressing rate of the different concentrations of ATP on the I_{K}^+ evoked using a 30 mV voltage was 3.51 \pm 3.8% (n=6) by 0.1 μM ATP, 12.58 \pm 4.62% (n=6) by 1 μM ATP, and 44.49 \pm 9.76% (n=9) by 10 μM ATP. The outward current was totally blocked by 100 μM (n=6) or 1 mM ATP (n=6). The concentration-response curve was fitted using the logistic equation (Figure 3B). The inhibition concentration (IC_{50}) was 12.88 \pm 1.58 μM .

Suppression of I_{K}^+ by ATP is voltage-dependent

From the I/V curve (Figure 2A), it was observed that I_{K}^+ was activated at approximately -30 mV and the amplitude was saturated at approximately 30 mV. ATP inhibited the K⁺ current, but did not change the activation or saturation voltage of I_{K}^+ . To determine whether the membrane potential had any effect on the inhibition of ATP (Figure 4), a 2-way ANOVA test was

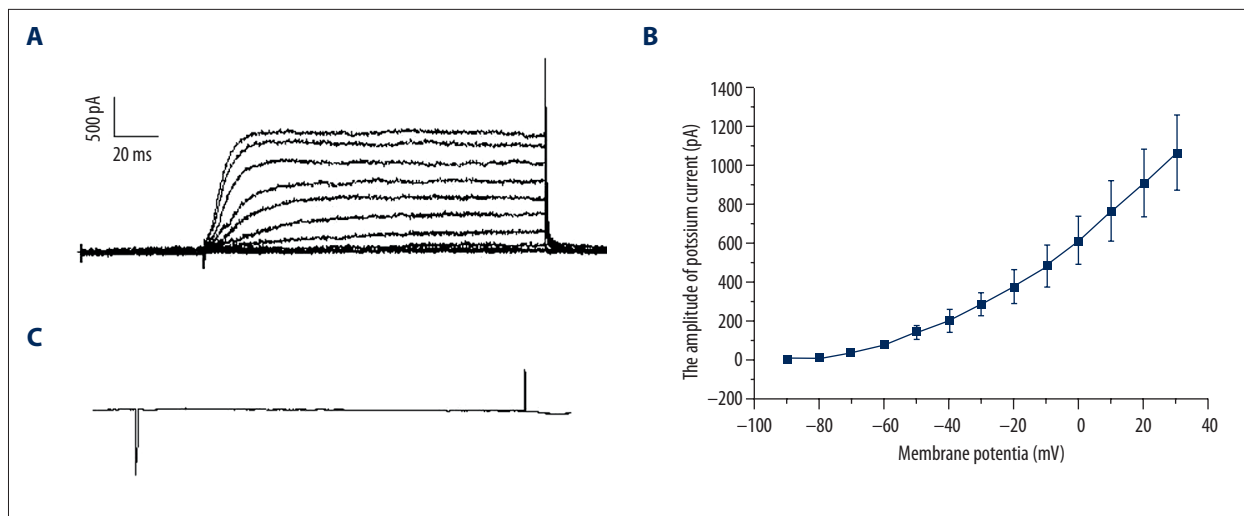


Figure 2. Outward K^+ current recorded from a single isolated Hensen's cell. (A) Typical raw data evoked by a voltage step from -90 to $+60$ mV (10 mV step). (B) I/V curve of I_{K^+} . (C) 40 mM TEA could block the I_{K^+} .

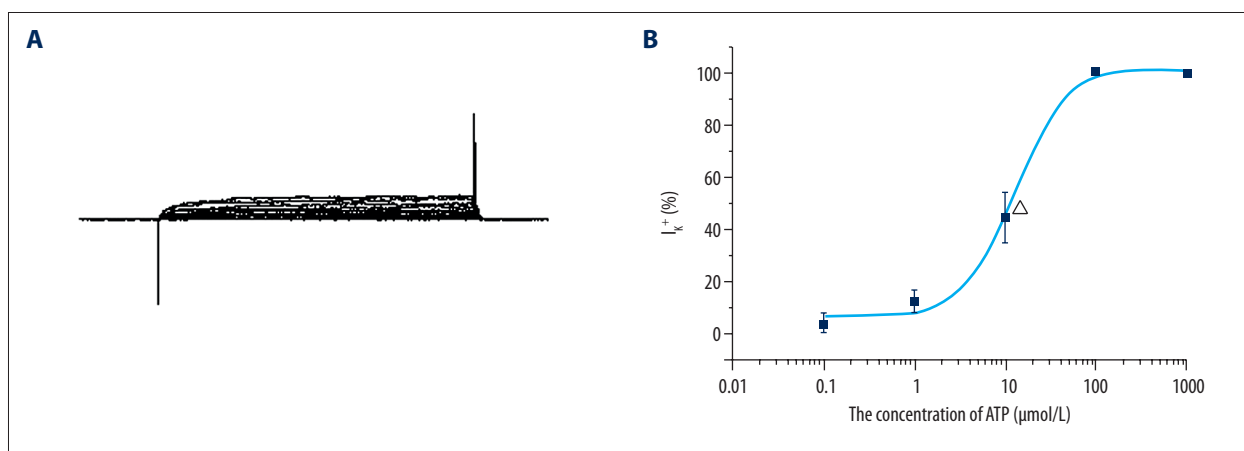


Figure 3. ATP could block the I_{K^+} evoked by voltage steps (-90 to $+60$ mV). (A) I_{K^+} could be blocked by low concentrations of ATP. (B) the concentration-response curve of the ATP suppression effect on I_{K^+} was fitted with the logistic equation. Note that the IC_{50} was $12.88 \pm 1.58 \mu\text{M}$.

used to test the suppression rate of I_{K^+} with different clamp voltages. We observed that the change in membrane potential had a significant interaction with the inhibition of ATP ($F=46.95$, $P<0.05$) compared to the average I_{K^+} in the absence of ATP (Figure 4). Membrane potential significantly changed the amplitude of I_{K^+} vs. the control group ($n=6$, $F=50.03$, $P<0.05$), and ATP significantly depressed the I_{K^+} at increased concentrations ($F=40.80$, $P<0.05$). The change of membrane potential had a significant interaction with the inhibition of ATP ($F=46.95$, $P<0.05$).

A K^+ -dependent inward current is invoked by a high concentration of ATP and blocked by nifedipine

At concentrations of 100 μM , 1 mM, and 10 mM, ATP evoked an averaged inward current of 200 pA, 350 pA, and 470 pA

(18 from 25 cells), respectively (Figure 5). No ATP-induced current could be recorded after I_{K^+} was blocked using intracellular Cs^+ (140 mM) or extracellular TEA (40 mM). This suggests that the inward current probably passes through the I_{K^+} channels ($n = 6$) that are sensitive to ATP. The inward current could be reversely blocked using suramin (100 μM), a purinergic receptor antagonist, suggesting that the current was triggered by ATP through purinergic receptors. The inward current was also blocked by nifedipine (10 μM), a calcium channel blocker.

Nifedipine has a possible prophylactic effect against NIHL

The animals were randomly divided into 4 groups: 1) perfused with artificial perilymph solution (APS); 2) perfused with APS containing 0.5 $\mu\text{mol/L}$ nifedipine (NIF); 3) perfused with APS and exposed to noise (APS+noise); and 4) perfused with

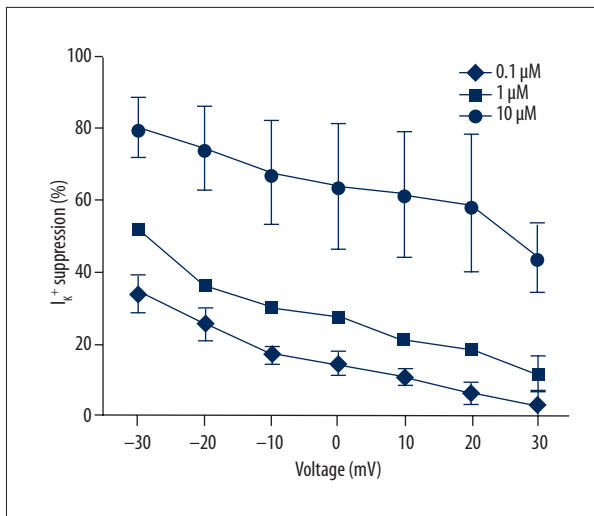


Figure 4. ATP-induced I_k⁺ suppression (%)

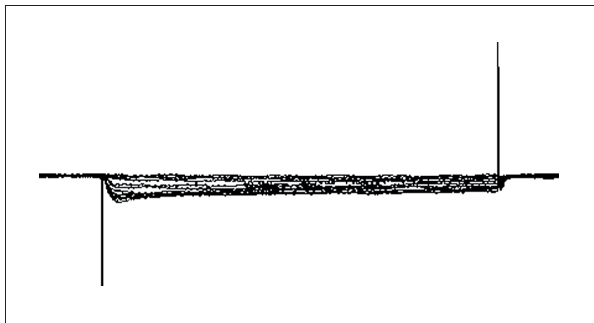


Figure 5. ATP-induced inward currents from isolated Hensen's cells.

APS containing 0.5 μmol/L nifedipine and exposed to noise (NIF+noise). There were no significant differences in the CAP thresholds or CM amplitudes before and after perfusion in the APS group ($P>0.05$). A rise in the CAP threshold and a reduction in the CM amplitude after perfusion were found in the other 3 groups ($P<0.01$). The increase in CAP thresholds after perfusion of these 3 groups was 28, 43.5, and 20 dB SPL, respectively (Figure 6). The reduction of CM amplitudes in the same 3 groups was 70.3%, 37.1%, and 21.1%, respectively (Figure 7). After perfusion with APS containing nifedipine and exposure to noise, the rise in the CAP threshold and the reduction in CM were lower than when exposed to noise alone ($P<0.01$).

Discussion

The present results show that low concentrations of ATP exerted a suppressive effect on the voltage-gated K⁺ currents (I_k⁺) in isolated adult Hensen's cells. This effect was voltage-dependent and suggests that ATP directly interacted with the K⁺ channel pore by binding to a site partway across the membrane electric field, although the site of action could not be

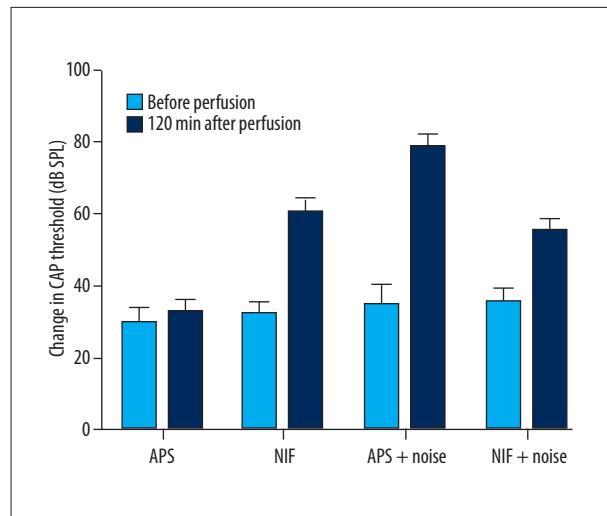


Figure 6. Change in CAP threshold (dB SPL, $x \pm SD$, $n=10$). Rises in the CAP thresholds after perfusion were observed in NIF, APS+noise, and NIF+noise groups ($P<0.01$). After perfusion with APS containing nifedipine and exposure to noise (NIF+noise), the rise in the CAP threshold was lower than those observed following exposure to noise alone ($P<0.01$).

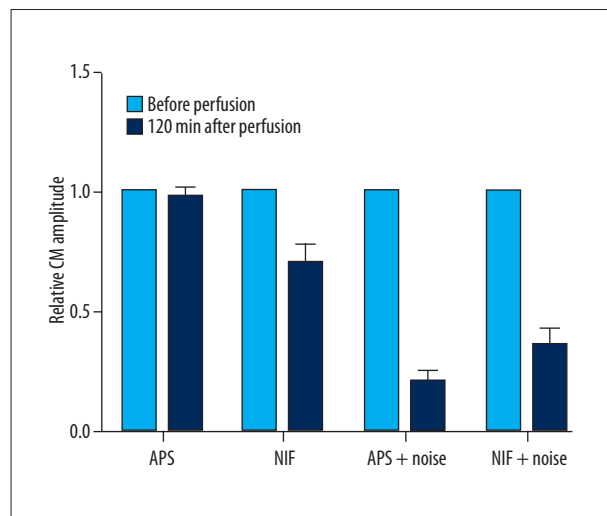


Figure 7. Relative CM amplitude ($n=10$). The reductions in the CM amplitudes after perfusion were observed in NIF, APS+noise, and NIF+noise groups ($P<0.01$). After perfusion with APS containing nifedipine and exposure to noise (NIF+noise), the reduction in the CM amplitudes was lower than those observed following exposure to noise alone ($P<0.01$).

determined in this experiment. The inhibition effect recovered quickly after ATP was removed, suggesting that the inhibition was reversible. This finding is consistent with the effect of ATP on the cells of the stria vascularis [17].

Previous studies have demonstrated that Hensen's cells have a resting potential ranging from -60 to -100 mV [18,19]. A relatively high K^+ concentration is required in the extracellular solution to maintain this resting potential. One possible source for the high K^+ concentration in the endolymph could be the rich blood supply of the stria vascularis [20]. As a high concentration of extracellular K^+ can depolarize Hensen's cells [21], an outward K^+ current during hyperpolarization could increase the concentration of K^+ in the endolymph and maintain a high level of EP. Any factor that influences EP will result in hearing loss. Evidence suggests that ATP receptors are abundant in the organ of Corti of guinea pigs, including Hensen's cells, outer hair cells, Deiter's cells, pillar cells, and inner hair cells [22]. The introduction of extracellular ATP into the endolymphatic compartment of the guinea pig cochlea has a significant dose-dependent suppressive effect on EP and CM. In this experiment, ATP inhibited the outward flow of K^+ from Hensen's cells and this could decrease EP and CM [23], implying that ATP may modulate the outward K^+ current of Hensen's cells to maintain the high concentration of K^+ in the endolymph and modulate hearing.

ATP is known to be the basis of Ca^{2+} current in numerous tissues, including the cochlea [24]. In this study, a high concentration of ATP evoked an inward current that could be blocked by nifedipine (i.e., the blockage of L-type calcium channels), suggesting that a high likelihood that the inward current was carried by Ca^{2+} ; moreover, the inward current could be blocked by suramin, a purinergic receptor antagonist. This suggests that the inward current is reversed by ATP through purinergic receptors that could include a ligand-gated channel (P_{2X}) or a G-protein-related channel (P_{2Y}). As the inward current was carried by Ca^{2+} , it is likely to be a G-protein-related channel.

After the cochlea was perfused with an APS, which contained nifedipine, and exposed to noise, the amplitude rise in the CAP

threshold and reduction in CM were lower than with noise alone, showing that nifedipine may have protective effects on NIHL, which is consistent with previous experimental results [15]. Previous studies have demonstrated that endolymphatic Ca^{2+} plays a considerable role in hearing [25]. Noise exposure induces increased Ca^{2+} concentration along with linear auditory brainstem response (ABR) threshold shifts [18]. When Ca^{2+} channel blocker and noise exposure were introduced simultaneously, it was subtractive instead of simply additive, with a certain level of "counteraction" [15]. ATP increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Hensen's cells, and the influx of extracellular Ca^{2+} is essentially induced by increases of $[Ca^{2+}]_i$ [26]. Calcium imbalance, in turn, damages the mitochondrial function and the increase of $[Ca^{2+}]_i$ causes local vasoconstriction, resulting in hypoperfusion of the inner ear [27]. In most cells, the reduction of ATP is caused by mitochondrial damage and dysemia [28]. A study showed that the depletion of ATP, induced by traumatic noise exposure, could evoke hair cell death and was associated with Ca^{2+} influx [14]. In this study, we found that nifedipine could also block the inward Ca^{2+} current induced by ATP. Many studies have shown that the accretion of intracellular calcium was associated with hearing loss and the calcium channel blockers could improve hearing loss caused by factors such as age, noise, and chemotherapy [29–31]. These findings indirectly demonstrate that nifedipine may have a possible prophylactic effect against NIHL.

Conclusions

In this study, we demonstrated that: 1) ATP inhibited the voltage-gated K^+ channel. The inward current evoked by ATP at high concentrations was I_K^+ -dependent and was triggered by ATP through a purinergic receptor. 2) Nifedipine may have a prophylactic effect against NIHL.

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