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Nitric oxide influences potassium currents in inner hair cells isolated from guinea-pig cochlea

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ABSTRACT

Objective: Nitric oxide (NO) is a diffusible second messenger, which regulates neurotransmission, serving as the principal endothelium-derived relaxing factor. NO also acts as an ion channel modulator. Nitric oxide synthase (NOS) has been identified in the inner ear, although its physiological role remains unclear. In the present study, the effects of NO onto K currents in inner hair cells (IHCs) were investigated.

Methods: IHCs were acutely isolated and K currents were recorded by conventional whole-cell voltage-clamp recordings. NO donors sodium nitroprusside (SNP) were applied directly to the cells.

Results: In 1 mM SNP solutions, the amplitude of outward K currents ($I_{K,f}$ and $I_{K,s}$) reversely decreased; however, fast activation kinetics was preserved. In the current–voltage relationship curves, the maximal slope conductances were 53.2 nS and 44.2 nS in control solutions and 1 mM SNP solutions, respectively. At the membrane potential of +110 mV, the amplitudes of outward currents were 9.2 ± 2.9 nA in control solutions and 7.3 ± 2.7 nA in 1 mM SNP solutions, showing a significant difference.

Conclusion: NO acts as a K channel modulator in IHCs. A fast K current suppression may account for the high-frequency hearing impairment by the prevention of fast repolarization.

1. Introduction

Nitric oxide (NO) is a diffusible second messenger, expressed in the nervous and cardiovascular systems, and regulates neurotransmission, serving as the principal endothelium-derived relaxing factor [1,2]. Experimental evidence indicates that, once synthesized, NO rapidly diffuses into neighboring cells, thus regulating their activity. NO has been proposed to modify mechanisms underlying several physiological responses [3], acting as an ion channel modulator [4,5].

The enzyme that forms NO from arginine, nitric oxide synthase (NOS), has been identified in the inner ear. In the cochlea, afferent and efferent nerve fibers and hair cells contain NOS [6–8]. Hess et al. [9] found NOS I and III in inner hair cells (IHCs) and outer hair cells (OHCs) of mouse cochlea. NADPH diaphorase, one of the other enzymes that synthesize NO, has been found in spiral ganglion cells [10], lateral wall and neuroepithelium [11] in the cochlea, although its physiological role remains unclear.

In the mammalian cochlea, there are two types of hair cell that serve distinct functions and receive characteristic patterns of innervation. Inner hair cells (IHCs) receive nearly all afferent innervations and are primary acoustic transducers. The three IHC potassium currents are distinguishable by their pharmacology and their activation kinetics [12–14]. The fast activating current, $I_{K,f}$, is blocked by tetraethylammonium (TEA) but is resistant to 4-aminopyridine (4-AP). $I_{K,s}$ is activated more slowly on depolarization and is blocked by 4-AP but not by TEA. Another potassium current, $I_{K,n}$, is already activated at the resting potential of the cell and thus determines the resting membrane potential and membrane constant. Potassium currents are known to participate in the repolarization and discharge behaviors of action potentials in neurons [15,16]; therefore, changes in IHC potassium currents may affect the IHC presynaptic function. Thus, potassium currents are crucial for maintaining the physiological functions of the cell.

In the present study, in order to determine the underlying mechanisms for the NO-induced changes in mammalian auditory hair cells' excitability, IHCs from guinea-pig cochlea were acutely isolated and their potassium currents were identified. Since NO is a

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gas and is unstable in solution, various drugs containing NO in their structure have been used because of their ability to donate NO to tissue [17]. The effects of NO on potassium currents were studied by applying the NO donor sodium nitroprusside (SNP).

2. Materials and methods

2.1. Preparation of isolated IHCs

An adult albino guinea pig (200–350 g) was killed by rapid cervical dislocation, both bullae were removed and the cochlea was exposed. The cochlea, fused to the bulla, was placed in a Ca^{2+}-free external solution (mM: 142 NaCl, 4 KCl, 3 $MgCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH). The otic capsule was opened, allowing removal of the organ of Corti attached to the modiolus. The organ of Corti was treated with trypsin (0.5 mg/ml, T-4665, Sigma, MO, St Louis) for 12 min, and gentle mechanical trituration was carried out. Trypsin was rinsed from the specimen by perfusion with a standard external solution (mM: 142 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH) for at least 10 min before starting any experiments. The most important landmarks for identifying IHCs are a tight neck and angle between the cuticular plate and the axis of the cell as described previously [18–20]. In the present study, IHCs were isolated from all turns of the cochlea. IHCs in the basal turn are indistinguishable from those in the apical turn based on shape.

2.2. Recording procedures

Membrane currents were measured by conventional whole-cell voltage-clamp recording using an EPC-10 (HEKA, Lambrecht, Germany). Data acquisition was controlled using the software PatchMaster (HEKA, Lambrecht, Germany). Recording electrodes were pulled with a two-stage vertical puller (PP830 Narishige, Tokyo, Japan) using 1.5-mm-external-diameter borosilicate glass (GC-1.5, Narishige, Tokyo) filled with an internal solution (mM: 144 KCl, 2 $MgCl_2$, 1 NaH_2PO_4, 8 Na_2HPO_4, 2 ATP, 3 D-glucose, 0.5 EGTA, adjusted to pH 7.4 with KOH). Pipettes showed resistance of 4–8 MΩ in the bath and were coated with ski wax (Tour-DIA, DIAWax, Otaru, Japan) to minimize capacitance. Automatic adjustment of capacitance transients cancelation and series resistance compensation were applied during all whole-cell experiments. The cell's capacitance was 15.8 ± 3.5 pF (mean ± s.d.) and the series resistance was 17.1 ± 8.7 MΩ (n = 12).

Sodium nitroprusside (SNP, S-0501, Sigma–Aldrich, St. Louis, MO) was applied under pressure (Pressure micro-injector: PMI-200, Dagan, Minneapolis) using pipettes with a tip diameter of 2–4 μm positioned around 50 μm from the IHCs. Cells were continuously perfused with external saline and all experiments were performed at room temperature (20–25 °C).

2.3. Animal care

The experimental design was reviewed and approved (Accession No. A25-003-0) by the Animal Care and Use Committee, Kyushu University. All procedures were conducted in accordance with the Guidelines for Animal Care and Use Committee, Kyushu University.

3. Results

Membrane currents in response to hyperpolarizing and depolarizing voltage steps from a holding potential of −60 mV were recorded from IHCs. In standard control solutions, IHCs showed outwardly rectifying K currents (constituted of fast activating $I_{K,f}$ and slow activating $I_{K,s}$) in response to depolarizing voltage pulses, with only a slight inward current ($I_{K,n}$) when hyperpolarized (Fig. 1A, left). To examine the effects of NO, SNP was applied directly to the isolated IHCs. In the solution of 1 mM SNP, the amplitude of outward K currents decreased by 13% (Fig. 1A, middle), and the reduction induced by SNP was reversed by washing with control solutions (Fig. 1A, right). An initial peak was reduced; therefore the fast activating $I_{K,f}$ was mainly affected by SNP. In spite of the amplitude reduction, fast activation kinetics was preserved, that is, displaying the fast rising phase in the outward currents (Fig. 1B). In the membrane potential of +60 mV, the initial rising phase could be fitted by double exponential curves in control and SNP solutions (Fig. 1C). Fast time constants (τ_{fast}) were 0.349 ±0.085 ms and 0.344 ± 0.078 ms (N = 5) in control solutions and in SNP solutions, respectively, showing no statistical differences (Student's t-test).

Fig. 2 presents the steady-state current–voltage relationship (I–V) measured at the end of each 100-ms command step for all 10 cells in control solutions and 1 mM SNP solutions. Both curves showed pronounced outward rectification with maximal slope conductances of 53.2 nS and 44.2 nS in control solutions and SNP solutions, respectively. Outward currents were clearly affected; however, inward currents ($I_{K,n}$) did not change by SNP application.

The amplitudes of outward currents at +110 mV were 9.2 ± 2.9 nA (mean ± s.d.) in control solutions and 7.3 ± 2.7 nA in 1 mM SNP solutions (Fig. 3), showing a significant difference (Mann–Whitney U-test, $p < 0.05$).

4. Discussion

The effects of NO on the K channels in cochlear IHCs were studied by using the NO donor SNP. SNP reversely decreased the current amplitudes without affecting the fast activation phase. NO-dependent regulation of K currents varies from suppression to upregulation, depending on the cells or tissue types. In type I hair cells from rat semicircular canals, the addition of NO donors to the external solution reduced the instantaneous currents evoked by voltage steps from −67 mV, which were principally carried by $I_{K,L}$ channels [21]. In hair cells from frog saccules, Ca^{2+}-activated K^+ currents were reversibly enhanced after application of NO by increasing the open probability of BK channels [22]. In posterior pituitary nerve terminals, NO enhanced Ca^{2+}-activated K^+ channel activity by activating guanylate cyclase (GC) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) [23].

The actions of NO may involve direct nitrosylation of the channel since dithiothreitol, a thiol reducing reagent, reversed the effects of NO on whole-cell and single-channel currents [22]. Chen et al. [21], however, demonstrated that NO's effect is mediated by intracellular second messengers because perfusion of the cell with 1 mM SNP greatly reduced all channel activity when the patch was cell-attached, but excision of the patch restored channel activity, despite continued perfusion with SNP. This suggests that the SNP effect is mediated by soluble second messengers that are no longer present in excised patches. The full effect of nitroglycerin, another NO donor, took minutes to develop, ranging from 1 to 4 min, suggesting that NO's effect is mediated by a second-messenger cascade [21]. Ca^{2+}-dependent NO synthase has been reported in hair cells and nerve terminals in the vestibular epithelium. In some systems, NO's effects are mediated by cGMP, produced as a consequence of the NO activation of GC. Ionic channel modulation by NO can be produced indirectly through second messengers involving the activation of the soluble form of GC and increased levels of cGMP [24,25]. NO exerts many of its actions *via* several interrelated redox forms to produce direct and distinct reactivities on proteins [26]. It has been demonstrated that the regulation of protein functions by alteration of the redox state through reactions of vicinal thiols, which serve as allosteric modulators of ion channels [27]. NOS was colocalized with sGC and cGMP in the