Release and Elementary Mechanisms of Nitric Oxide in Hair Cells

Ping Lv,1,* Adrian Rodríguez-Contreras,1,* Hyo Jeong Kim,1 Jun Zhu,2 Dongguang Wei,1 Sihn Choong-Ryoul,1 Emily Eastwood,1 Karen Mu,1 Snezana Levic,1 Haitao Song,1 Petrov Y. Yevgenyi,1 Peter J. S. Smith,3 and Ebenezer N. Yamoah1,3

1Center for Neuroscience, Department of Anesthesiology and Pain Medicine, Program in Communication Science, University of California, Davis, Davis, California; 2Department of Nephrology, Xijing Hospital, Fourth Military Medical University, Xi’an, China; and 3BioCurrent Research Center, Marine Biological Laboratory, Woods Hole, Massachusetts

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INTRODUCTION

Hair cells of vestibular and auditory systems are neuroepithelia cells and, like neurons, their electrical outputs are governed by intrinsic membrane properties and extrinsic synaptic inputs. However, unlike other primary sensory systems in which the stimulus is processed through a cascade of biochemical reactions, stimulation of hair cells is coupled directly to mechanical stimuli, making them ideally prone to mechanical damage (Hudspeth and Logothetis 2000; Yoshida et al. 2001; Hess et al. 1998; Lysakowski and Singer 2000), raising the possibility that evolutionary preservation of the NO pathway is of vital importance to inner ear functions. Indeed, measurable amounts of NO release from the organ of Corti has been reported and the level is increased after noise exposure. Moreover, it is unknown whether the increase in NO levels following noise exposure serves a protective or pathological role (Shi et al. 2002). Nonetheless, the physiological significance of NO in hair cells may be derived from the effects of the reactive molecular messenger on their ionic conductances, although the net action of NO on ionic currents in hair cells is complex. On one hand, it has been demonstrated that NO inhibits a large conductance outward rectifier K+ current (\(I_{\text{K,L}}\)) in type I hair cells in rat crista ampullaris (Chen and Eatock 2000), suggesting that NO produces an increase in membrane excitability. In contrast, NO reduces the magnitude of Ca2+ currents (Almanza et al. 2007), yielding an opposite effect on the membrane potential.

To further understand the sources and effects of NO on hair cells, we determined whether the hair cell release NO and identified its elementary mechanisms of actions. Here, we show that NO is released by the sensory epithelia and by ACh. On release NO regulates Ca2+ and K+ channels in hair cells. Additionally, we provide evidence to demonstrate that NO modulates Ca2+ and K+ channels by direct nitrosylation.

feedback from the brain stem through activation of \(\alpha 9\alpha 10\) nicotinic acetylcholine (ACh) receptors and a small conductance Ca2+-activated K+ current (Chen and Eatock 2000; Elgoyhen et al. 2001; Katz et al. 2004; Yoshida et al. 2000; Yuhas and Fuchs 1999). Whereas release of ACh at the efferent nerve terminals elicits a fast membrane hyperpolarization (Housley et al. 2006), prolonged stimulation of efferent nerves produces further delayed inhibition, whose mechanism remains elusive (Sridhar et al. 1997).

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 (Bredt et al. 1990; Stamler and Loscalzo 1992). The enzyme that forms NO from arginine, nitric oxide synthase (NOS), has been identified in the inner ear, although its physiological role remains unclear. The spiral modiolar artery may be one of the major sources of endogenous production of NO (Jiang et al. 2004). However, NO is expressed not only in hair cells but also in afferent and efferent nerve terminals in a variety of animal models ranging from amphibians to mammals (Fessenden et al. 1994; Flores et al. 2001; Hess et al. 1998; Lysakowski and Singer 2000), raising the possibility that evolutionary preservation of the NO pathway is of vital importance to inner ear functions. Indeed, measurable amounts of NO release from the organ of Corti have been reported and the level is increased after noise exposure. Moreover, it is unknown whether the increase in NO levels following noise exposure serves a protective or pathological role (Shi et al. 2002). Nonetheless, the physiological significance of NO in hair cells may be derived from the effects of the reactive molecular messenger on their ionic conductances, although the net action of NO on ionic currents in hair cells is complex. On one hand, it has been demonstrated that NO inhibits a large conductance outward rectifier K+ current (\(I_{\text{K,L}}\)) in type I hair cells in rat crista ampullaris (Chen and Eatock 2000), suggesting that NO produces an increase in membrane excitability. In contrast, NO reduces the magnitude of Ca2+ currents (Almanza et al. 2007), yielding an opposite effect on the membrane potential.

To further understand the sources and effects of NO on hair cells, we determined whether the hair cell release NO and identified its elementary mechanisms of actions. Here, we show that NO is released by the sensory epithelia and by ACh. On release NO regulates Ca2+ and K+ channels in hair cells. Additionally, we provide evidence to demonstrate that NO modulates Ca2+ and K+ channels by direct nitrosylation.
Moreover, NO further regulates Ca\(^{2+}\) channels through NO-mediated activation of protein kinase G.

**METHODS**

**Tissue preparation**

The bullfrog saccule was isolated as previously described (Rodriguez-Contreras and Yamoah 2001). The protocol was approved by the University of California, Davis Animal Research Services IACUC committee. Frogs were killed and inner ears were quickly removed and placed in oxygenated low-Ca\(^{2+}\)/frogl Ringer solution (mM): 110 NaCl, 2 KCl, 3 d-glucose, and 0.1 CaCl\(_2\). The saccular macula was isolated and incubated in frog saline containing 50 \(\mu\)g/ml protease (type XXIV; Sigma, St. Louis, MO) for 20 min. The tissue was then washed and transferred to frog saline containing 1 mg/ml bovine serum albumin (BSA; Sigma) and 2 mg/ml DNase I (Worthington, Lakewood, NJ) for 10 min. The otolithic membrane was excised and the sensory epithelium placed in a recording chamber using cell Tak to secure the tissue at the bottom of the chamber. For experiments in which isolated cells were required, the macular tight junctions in the sacculare were disrupted by exposing the perilymphatic surface to 4 mM ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N\(_2\)N\(_3\)N\(_4\)N\(_5\)-tetraacetic acid (EGTA) for 15 min. After washing the preparation with fresh saline, the saccular macula was isolated and incubated in frog saline containing 50 \(\mu\)g/ml protease (type XXIV; Sigma) for 20 min. The tissue was then washed and transferred to frog saline containing 1 mg/ml BSA and 2 mg/ml DNase I for 10 min. This procedure has been used previously (Chabbert 1997) and differs from more severe enzymatic treatments that can alter ionic conductances (Armstrong and Roberts 1998). The otolithic membrane was excised and hair cells were dissociated from the macula by use of an eyelash. Hair cells were allowed to settle onto the bottom of the recording chamber (coated with lectin concavalin-A) for 20 min prior to patch-clamp recording.

**Preparation of NO microsensors**

Carbon fiber electrodes were constructed as previously described (Kumar et al. 2001; Porterfield et al. 2001), by inserting a 5 \(\mu\)m carbon fiber (Amoco, Greenville, SC) through a borosilicate glass pipette (OD 1.5 mm; ID 0.86 mm; World Precision Instruments, Sarasota, FL), using acetone as a lubricant. The electrode was dried at 110°C for 10 min and then pulled using a horizontal puller (P97; Sutter Instrument, Novato, CA). The fiber was retained and sealed within the pulled pipette with Epoxytite (Epoxytite, Westerville, OH) and cured at 110°C for 4 h. Once the epoxylite had cured, the electrode was backfilled with graphite-epoxy paste (PX-grade Graphite-e-poxy; Dylon Industries, Cleveland, OH). To establish electrical contact between the carbon fiber and a preamplifier, a copper wire (diameter: \(\sim 700 \mu\m\)) was inserted into the glass electrode in contact with the graphite-epoxy paste. The graphite-epoxy paste was polymerized at 110°C overnight. The tip of the carbon fiber was trimmed and beveled at a 30° angle and a final tip diameter of about 2–3 \(\mu\m\) was obtained. To make the electrode selective for the oxidation of NO, the carbon fiber was modified using the procedure described (Friedemann et al. 1996). The carbon fiber was coated with Nafion (5% in aliphatic alcohol; Aldrich, Milwaukee, WI) and o-phenylenediamine (o-PD; Sigma). Nafion is a polysulfonated Teflon that carries negative charge and repels electrochemically active anions (e.g., nitrate, nitrite, and ascorbate). o-PD confers selectivity to NO oxidation by size exclusion of noncharged interferents such as electrochemically active catecholamines. First, the carbon fiber was coated with Nafion and dried at 110°C for 10 min and, using this procedure, was repeated twice. Next, the Nafion-coated carbon fiber was plated with a 5 \(\mu\m\) o-PD plating solution consisting of 0.1 mM ascorbic acid in 100 mM phosphate buffer saline (PBS, pH 7.4). The plating voltage was a constant +0.9 V applied until desirable noise characteristic was obtained. The NO-selective electrode was operated with an Ag/AgCl return electrode that completed the circuit in solution though a 3 M KCl/3% agar bridge. The polarization voltage used was 0.9 V and the electrode was operated in self-referencing mode, as described for self-referencing NO and O\(_2\) electrodes (Kumar et al. 2001; Land et al. 1999).

**Calibration of NO electrodes**

A stock of saturated NO solution was prepared fresh daily. First, frog saline was made O\(_2\)-free by bubbling the solution in a vacuum-sealed container with O\(_2\)-free argon for 45 min. The solution was then bubbled with NO gas for 30 min. The pH of the solution was adjusted to 7.4. The concentration of NO obtained using this protocol was about 2 mM (Ahmed et al. 2001; Friedemann et al. 1996). The stock solution was diluted into several series and the sensitivity of the electrode tested. Five electrodes were constructed and their sensitivity for NO was 0.77 \pm 0.15 pA/\mu M (n = 5). In contrast, the sensitivity of the electrodes to sodium nitrite was roughly 600-fold less than that to NO (sensitivity for nitrites was 1.28 pA/\mu M). Despite the variations in the sensitivity of the electrodes, they responded well to changes in NO concentrations in a linear fashion (Porterfield et al. 2001; see Fig. 1). To reduce inherent drifts associated with polargraphic electrodes and to increase the sensitivity of the NO electrodes, the electrodes were used in a self-referencing mode (Smith et al. 1999). Using the release of NO from S-nitroso-N-acetylpenicillamine (SNAP) in a source micropipette to test for the sensitivity of the NO electrodes, we measured the gradient generated by the source (10 mM SNAP in PBS in \(\sim 10 \mu\m\) diameter micropipette) and thus established that the NO selective electrodes were true detectors of NO flux (Porterfield et al. 2001; Shafer et al. 1998; Fig. 1).

Measurements of NO fluxes were performed using the intact saccular epithelium. The tissue was placed on a Zeiss and/or Olympus (BX50WI) upright microscope fitted with a stage plate on which the head stage and translational motion control system were mounted. Motion control allows nanometer resolution of movement of the head stage using linear translation stages at 0.3 Hz and in static mode (Smith et al. 1999). The amplifier, motion control, and data acquisition system were built with custom-written software (IonView, BioCurrent Research Center, Woods Hole, MA). Data were collected at a rate of 1,000 events/s and signal-averaged to 10 values consisting of 166 data points/position. Values obtained over the period of electrode movement were excluded (first third discarded).

**Whole cell recordings**

Recordings were performed using an Axopatch 200B patch clamp amplifier (Molecular Devices, Union City, CA) interfaced to a personal computer. Voltage and current commands were generated and data collected using custom-written software. Patch pipettes were fabricated from borosilicate glass capillaries (World Precision Instruments) on a four-step horizontal Flaming-Brown microelectrode puller (Sutter Instrument) and coated with Sylgard 184 (Dow-Corning, Midland, MI) to within 100 \(\mu\m\) of the tip and fire-polished before use. To record Ca\(^{2+}\) currents, outward K\(^{+}\) currents were blocked with tetraethylammonium chloride (TEA-Cl), 4-amino pyridine (4-AP), and cesium (Cs\(^{+}\)) ions. Bath solution contained (in mM) 90 NaCl, 25 TEA-Cl, 5 4-AP, 5 CaCl\(_2\), 5 glucose, and 5 HEPES (pH 7.4). Whole cell Ca\(^{2+}\) currents were recorded using perforated patch electrodes (resistances 2–4 M\(\Omega\)) to minimize washout of intracellular molecules. The tips of electrodes were filled with a solution containing (in mM) 130 CsCl and 5 HEPES (pH 7.3 with CsOH). To gain electrical access to the cell, electrodes were backfilled with solution containing (in mM) 130 CsCl, 1 CaCl\(_2\), 5 HEPES, and amphotericin 200 \(\mu\)g/ml (pH 7.3 with CsOH) (Korn et al. 1991). To ensure that recordings were in the perforated-patch mode instead of whole cell mode, the backfilled solution of the patch electrode contained 1 mM Ca\(^{2+}\). A switch from
the perforated to whole cell mode resulted in cell death because of Ca$^{2+}$ toxicity. Series resistance (5–10 MΩ) was compensated (nominally 50–60%). Liquid-junction potentials were <2 mV and were not corrected. Ca$^{2+}$ current records were amplified and filtered at 2–5 kHz with a low-pass Bessel filter and digitized at 10 kHz with a Digidata interface (Axon Instruments) controlled by custom-written software.

To record whole cell K$^+$ currents, the pipettes were filled with a solution containing (in mM): 130 KCl, 0.1 CaCl$_2$, 5 MgATP, 1–5 EGTA, 10 HEPES, and 1 Na$_3$GTP, with a pH of 7.3. The external solution was (in mM): 3 KCl, 2 CaCl$_2$, 120 NaCl, 5 HEPES, and/or 5 4-AP, 2.5 TEA, pH 7.4 (NaOH). Data were stored on a personal computer. All experiments were performed at room temperature (≈21°C). The capacitance of the cell was calculated by integrating the area under an uncompensated capacitive-transient, elicited by a 20 mV hyperpolarizing pulse from a holding potential of −80 mV.

**Single-channel recordings**

The standard configurations for cell-attached single-channel of the patch-clamp techniques (Hamill et al. 1981) were used to record Ca$^{2+}$/K$^+$ channel currents from hair cells isolated from the bullfrog saccule. Quartz electrodes were pulled with a laser puller (P2000; Sutter Instrument). Single-channel recordings of membrane patches were held at −70 or −60 mV, and stepped to different depolarizing test pulses at frequencies between 0.2 and 0.5 Hz. Current traces were amplified and filtered using an eight-pole Bessel filter at 2 kHz and digitized at 10 kHz using custom-written software. Patch electrodes were filled with a Ca$^{2+}$ solution (50–70 mM) containing (in mM) 20 TEA-Cl, 5 4-AP, and 5 HEPES at pH 7.4 (adjusted with TEA-OH). N-Methyl-D-glucamine (NMG) was used to substitute for divalent Ca$^{2+}$ and to maintain an osmolality of about 280 mosmole. Stock solutions of 3-pyridinecarboxylic acid-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-(trifluoromethyl)phenyl)methyl ester (Bay K 8644, 100 mM) were made in DMSO and a final concentration of 5 µM was used. The bath solution contained (mM) 80 KCl, 3 D-glucose, 20 TEA-Cl, 5 4-AP, and 5 HEPES at pH 7.4 with KOH/HCl. The pipettes were filled with a solution containing (in mM): 110 KCl, 5 4-AP, 0.1 CaCl$_2$, 10 HEPES, and 10 glucose (pH 7.3). In all cases, liquid-junction potentials were measured and corrected as described previously (Rodriguez-Conteras et al. 2002). All experiments were carried out at room temperature (≈21°C).

**Chemicals and solutions**

Several chemicals were used. These include NO-nitro-D-arginine methyl ester and NO-nitro-$\alpha$-arginine methyl ester ($\alpha$-NAME and L-NAME). ACh; 8 nmol·m$^{-2}$·s$^{-1}$; 2-nitro-L-arginine methyl ester (L-NAME) and 8-nitro-D-arginine methyl ester (D-NAME).
\( \text{d-NAME, respectively; Sigma), dithiothreitol (DTT), a thiol reducing reagent, sodium nitroprusside (SNP, Sigma), 3-morpholinosydnonimine (SIN-1, Molecular Probes, Eugene, OR), and S-nitroso-N-acetylpenicillamine (SNAP, Molecular Probes), NO donors. All other chemicals were obtained from Sigma, unless otherwise noted.} \)

\[ \text{Data analysis} \]

Whole cell current amplitudes at varying test potentials were measured at the peak and steady-state levels using a peak and steady-state detection routine. For single-channel records, leakage and capacitative transient currents were subtracted by fitting a smooth template to null traces. Leak-subtracted current recordings were idealized using a half-height criterion (Colquhoun and Sigworth 1985). Transitions between closed and open levels were determined by using a threshold detection algorithm, which required that two data points exist above the half mean amplitude of the single-unit opening. The computer-detected openings were confirmed by visual inspection and sweeps with excessive noise were discarded. Amplitude histograms at a given test potential were generated and then fitted to a single Gaussian distribution using a Levenberg–Marquardt algorithm to obtain the mean and SD. At least five voltage steps and their corresponding single-channel currents were used to determine the unitary conductance. Single-channel current–voltage (I–V) relations were fitted by linear least-square regression lines and single-channel conductances obtained from the slope of the regression lines. Idealized records were used to construct ensemble-averaged currents, open probability, and histograms for the distributions of open and closed intervals. Curve fits and data analyses were performed using Origin software (MicroCal, Northampton, MA). Where appropriate, pooled data are presented as means ± SD.

\[ \text{RESULTS} \]

\[ \text{Release of NO by the frog saccule} \]

Using a variety of direct and indirect immunoreactive techniques, it has been established that the vestibular maculae and cochlea of vertebrates show distinct expression of nitric oxide synthase (NOS; Fessenden and Schacht 1998; Lysakowski and Singer 2000; Michel et al. 1999; Riemann and Reuss 1999; Singer and Lysakowski 1996). To determine the functional significance of activation of NOS, we first characterized and quantified NO production in the frog saccule using the self-referencing amperometric probe technique (Fig. 1A). Figure 1B illustrates experiments performed to examine the sensitivity of five NO-selective electrodes and to determine the release of NO fluxes from the saccular epithelia. The current generated at the electrode, polarized to 0.9 V, was measured differentially between two points at the background (≈2,000 μm) from the sensory epithelia and at the tissue (≈2 μm from the tissue). Using the Fick equation as described (Kumar et al. 2001; Land et al. 1999), we calculated NO flux, by measuring the static NO concentrations over a given distance within a gradient established by an artificial source (Fig. 1C) and the tissue as a source of NO release. As illustrated in Fig. 1D, which shows a representative example of the NO efflux (nmol·m−2·s−1), when the electrode was moved from the background to the saccule, there was an approximately 200-fold change in the NO efflux. Application of frog saline containing \( \text{L-NAME resulted in gradual attenuation of NO efflux, consistent with the inhibition of NOS. Baseline extrusion of NO from the frog saccule was reliably observed in 15 samples that were tested (115 ± 20 nmol·m−2·s−1, } n = 15). Moreover, \( \text{L-NAME inhibited the NO efflux to 2 ± 12 nmol·m−2·s−1} (n = 8). In contrast, application of the inert enantiomer (d-NAME) produced a mean net efflux of 159 ± 36 nmol·m−2·s−1 (n = 7), thus suggesting that the NO efflux from the frog saccule was NOS-specific. Next, we examined the effects of ACh on NO efflux from the saccule. As shown in Fig. 1E, application of ACh caused a rapid and sustained efflux of NO. The ACh-mediated rise in NO efflux (ACh-induced effects = 324 ± 105 nmol·m−2·s−1, compared with control = 114 ± 27 nmol·m−2·s−1, } n = 9, } P < 0.05), was also inhibited by \( \text{L-NAME, again suggesting the involvement of NOS in the ACh-mediated release of NO. As shown in the summary data (Fig. 1F), the ACh-mediated release was dependent on extracellular Ca2+ Replacemen}
and DTT, as well as the \( I-V \) relation generated by a series of voltage-clamped steps from holding potential of \(-70\) mV. In the presence of NO donors, the whole cell \( \text{Ca}^{2+} \) current was reduced by \(34.0 \pm 6.0\%\) by SNAP \((n = 11)\), \(31.2 \pm 4.1\%\) \((n = 9)\) by SNP, and \(35.8 \pm 5.3\%\) by NO of the total current \((n = 14)\). NO produced an approximately \(3.5\) mV rightward shift in the steady-state activation curve (Fig. 3D). A concentration–response curve was constructed for NO and a half-blocking concentration of about \(80\) nM and Hill coefficient of \(1\) were determined (Fig. 3E). Because NO solution is relatively unstable after exposure to atmospheric \(O_2\), we simulated the experimental conditions for the perfusion of NO in the recording chamber and then used the NO-selective electrode to determine the NO concentration that was exposed to hair cells versus the applied concentration. Aside from the reduction of the magnitude of the \( \text{Ca}^{2+} \) current, NO produced no measurable effect on the kinetics of the current. Moreover, consistent with the current-clamp data, NO produced a two-phased effect: an early DTT-mediated reversible effect and a late DTT-irreversible effect. As illustrated in Fig. 3B, cGMP mimicked the late effect of NO and, in the presence of KT-5823 (the specific inhibitor of protein kinase G), DTT fully reversed the actions of NO. The summary data are shown in Fig. 3F.

Even though reduction of the inward \( \text{Ca}^{2+} \) currents could reduce the excitability of hair cells and increase the threshold for the induction of membrane oscillations, enhancement of outward \( K^+ \) currents could produce similar outcomes through membrane repolarization. We recorded whole cell \( K^+ \) currents and determined that only the \( \text{Ca}^{2+} \)-dependent component was affected by NO (data not shown). We examined the effects of NO on the \( \text{Ca}^{2+} \)-activated \( K^+ \) (BK) current by blocking the voltage-gated \( K^+ \) currents with 4-AP (Armstrong and Roberts 2001; Hudspeth and Lewis 1988a). Figure 4A illustrates BK-current traces, which were elicited from a holding potential of \(-90\) mV and stepped to depolarizing potentials \((\Delta V = 5\) mV) recorded in both the absence and the presence of NO and DTT. The BK current was enhanced after application of NO and its effects were reversed by DTT. The \( I-V \) relationship in Fig. 4B provides a summary of the data. The increase in magnitude of the outward current could result from the masking effect of reduction of inward \( \text{Ca}^{2+} \) current. However, the increase in magnitude of the outward \( K^+ \) current was larger than could be ascribed to a reduction of NO-induced block of the \( \text{Ca}^{2+} \) current alone, suggesting that NO may modulate the BK current as well. We examined this further by subjecting the cells to brief prepulses \((-25\) ms) to activate the VGCC, followed by a sustained \((>500\) ms) depolarizing test pulse to activate the BK current. Shown in Fig. 4C, the results indicated that NO suppressed the VGCC and enhanced the BK current as well; the effects of NO on the BK current were reversed by DTT. Thus NO-modified ionic currents in hair cells suppress their excitability.

Mechanisms for NO modulations of single \( \text{Ca}^{2+} \) and BK currents

Hair cells in the frog saccule express multiple \( \text{Ca}^{2+} \) and BK channels (Armstrong and Roberts 2001; Rodriguez-Contreras and Yamoah 2001; Rodriguez-Contreras et al. 2002). To prevent contamination and masking effects of inward and outward currents at the whole cell current level, a reasonable approach to determine the mechanisms of NO effect is to examine the current at the single-channel level. Figure 5, A–C shows single-channel \( \text{Ca}^{2+} \) currents obtained from a cell-attached patch containing a nimodipine-insensitive channel. The patch pipette contained \(65\) mM \( \text{Ca}^{2+} \) and the patch was held at \(-70\) mV and stepped to \(-50\) mV. Because of the pharmacology of the channel and its slow-voltage activation as well as its conductance \((16.2\) pS; Fig. 5E), the channel was classified as a non L-type \( \text{Ca}^{2+} \) channel (Rodriguez-Contreras and Yamoah 2001). Shown in Fig. 5D are examples of amplitude histograms, used to generate the \( I-V \) relations (Fig. 5E). The effect of NO on the probability of the non L-type channel openings \((P_o)\) was robust and reversible by DTT (0.2 mM) \((mean P_o values at test potential of \(-50\) mV were: control, 0.18 \( \pm \) 0.07; after application of NO, 0.07 \( \pm \) 0.01; and after DTT, 0.20 \( \pm \) 0.05; \(n = 9\))). Similar results were obtained when SNAP (100 \( \mu M\)) was used \((mean P_o values at test potential of \(-50\) mV were: control, 0.21 \( \pm \) 0.05; after application of SNAP, 0.09 \( \pm \) 0.03; and after DTT, 0.19 \( \pm \) 0.03; \(n = 5\)).
As depicted in Fig. 6, NO produced a modest decline in the \(P_o\) of the L-type channel, compared with that of the non L-type channel. The single L-type Ca\(^{2+}\) channel fluctuations recorded from hair cells in the cell-attached configurations were reduced by application of NO (500 nM) and SNAP (100 

muM). Examples of the L-type Ca\(^{2+}\) current traces recorded from a holding potential of \(-70\) mV and step potential of \(-30\) mV are shown in Fig. 6, A–C. The \(I-V\) relation shown in Fig. 6E was generated using unitary current magnitudes derived from the amplitude histograms (Fig. 6D). The conductance of the channel (13.6 pS) and its sensitivity to Bay K 8644 (the DHP agonist) suggest that the channel belongs to the L-class of Ca\(^{2+}\) channels. As shown in the diary plot in Fig. 6F, the application of 1 

muM NO reduced the \(P_o\) of the L-type channel. Moreover, the extent of reduction of the \(P_o\) by NO and SNAP was similar (control \(P_o\): 0.16 ± 0.06; NO: 0.07 ± 0.02; \(n = 7\); \(P < 0.01\); control \(P_o\): 0.14 ± 0.09; SNAP: 0.04 ± 0.01; \(n = 6\); \(P < 0.01\)) and was reversed partially by DTT (after DTT \(P_o\): 0.11 ± 0.02 (\(n = 7\)) and 0.09 ± 0.03 (\(n = 6\)) NO and SNAP, respectively. In contrast to the non L-type channel, which was reversed readily by DTT even after 20 min of application of NO, the effect of NO on the L-type channel was irreversible after 10 min exposure to NO. The differential effects of NO on the non L and L-type channels can be noticed in patches containing two channel subtypes (Fig. 7). Thus NO-induced reduction of the macroscopic Ca\(^{2+}\) current is derived from diminution of the \(P_o\) at the unitary channel level.

Open times were abbreviated in NO-modified Ca\(^{2+}\) channels. Open times were fitted by using bi- or triexponential functions for both patches. Compared with the control in Fig. 8, time constants obtained from NO-modified patches were significantly abbreviated by exposure to 100 nM NO [e.g., 2.2 ± 0.01 ms (control) vs. 0.50 ± 0.02 ms (\(n = 4\); \(P < 0.05\))] in NO-modified patches, respectively. DTT (0.2 mM) restored the fast open-time constant (1.1 ± 1.5; \(n = 3\); \(P = 0.7\)). Also important, the fast open-time constant was eliminated in NO-modified L-type single-channel current kinetics.
The activity of single BK channels was recorded in the cell-attached mode with patch pipettes containing 110 mM K⁺/H11001. Similar to previous reports (Armstrong and Roberts 1998; Art et al. 1995; Hudspeth and Lewis 1988a; Roberts et al. 1990), a large conductance (BK) channel with unitary current magnitude of about 7 pA at a step potential of 20 mV was recorded from hair cells. Shown in Fig. 9A are exemplary consecutive current traces recorded from a holding potential of −50 mV and at step potential of 20 mV. Amplitude histograms were generated to determine the unitary current amplitudes (e.g., Fig. 9C). The corresponding unitary I–V relations yielded a conductance of 275 ± 6 pS (n = 7; Fig. 9D). In contrast to the effects of NO on the Ca²⁺ channels, application of NO to the BK channel resulted in a substantial increase in Pₒ of the channel (Fig. 9B). The diary plot of the Pₒ illustrates the pronounced effects of NO compared with control and the recovery that ensues after application of DTT (Fig. 9E). However, the unitary conductance remains unchanged in both the presence and the absence of NO and DTT.

DISCUSSION

This study provides the first direct evidence to demonstrate that the sensory epithelia of the frog saccule release NO, which is enhanced by ACh in a Ca²⁺-dependent manner. The electrophysiological consequences of the release of NO are a

FIG. 4. Whole cell recordings of Ca²⁺-activated K⁺ current in hair cells. The amplitude of outward Ca²⁺-activated K⁺ currents was increased by NO. A: outward K⁺ currents traces were elicited at a holding potential (~−90 mV), step depolarization of ~80 to 40 mV, with change in voltage of 5 mV. The current was recorded in the presence of 5 mM 4-aminoypyridine (4-AP) and tetraethylammonium (TEA) to suppress other outward K⁺ currents (see METHODS). The current was enhanced in response to increased external Ca²⁺ (data not shown). The Ca²⁺-activated K⁺ currents were visibly enhanced on application of NO (600 nM) and the effect was reversed by 1 mM DTT. B: the summary data (n = 9) of the I–V relationship for data obtained from control experiments (○), after application of NO (●), and reversal effects of 0.2 mM DTT (△). C: to confirm the enhancement effect of NO on the Ca²⁺-activated K⁺ current, hair cells were held at −70 mV and stepped briefly (25 ms) to −30 mV to activate the inward Ca²⁺ current and then stepped to 5 mV to activated the outward K⁺ current (n = 5). The increase in the outward current was independent of the reduction of inward current. DTT completely reversed the effect of NO on the K⁺ current but not the Ca²⁺ current.

FIG. 5. Single-channel Ca²⁺ current fluctuations in hair cells plummeted in NO but the effect was reversed by DTT. Representative single-channel traces recorded using 65 mM Ca²⁺ as the charge carrier of (A) control, (B) 2 min after application of 500 nM NO, and (C) 2 min after application of 0.2 mM DTT. The recordings were made in bath and pipette solutions containing 10 μM nimodipine. Ten consecutive traces are shown at the step potential indicated (~−50 mV) from a holding potential of ~−70 mV. D: examples of amplitude histograms (step voltage, ~−50 mV) used to generate the I–V relationships for control (left) and after DTT (right). E: the I–V relationship of control (○) and after DTT (●). The single-channel conductances were: control, 16.2 ± 0.8 pS (n = 6) and after DTT, 15.8 ± 1.3 pS (n = 5) (P = 0.6). The insensitivity of the channel to nimodipine and the conductance suggests that it belongs to the non L-type Ca²⁺ channel subtype (Rodriguez-Contreras and Yamoah 2001).
profound suppression of the baseline membrane oscillation and a reduction in membrane excitability in hair cells. The elementary mechanisms for NO-induced effects are derived from NO inhibition of the non L- and L-type Ca\(^{2+}\) channels by attenuation of the \(P_o\). Moreover, NO inhibited non L-type Ca\(^{2+}\) channels and exhibited a sparse pattern of opening, which is reversed through redox modulation with DTT. Additionally, the inhibitory effect of NO on Ca\(^{2+}\) channels is augmented by the enhanced activity (\(P_o\)) of the large conductance BK channels in hair cells. Since activation of voltage-gated Ca\(^{2+}\) channels in hair cells occurs at hyperpolarized potentials (~50 mV), and specifically the \(P_o\) of the non L-type channels is substantial at rest (Rodriguez-Contreras and Yamoah 2001), NO inhibited Ca\(^{2+}\) channels and activated BK channels promote reduction of the membrane excitability of hair cells, resulting in an increase in the threshold of activation. Our findings provide the underlying elementary mechanism for the effects of NO on hair cells and help to clarify universal and divergent viewpoints of previous studies on NO-mediated actions (Almanza et al. 2007; Chen and Eatock 2000; Vega et al. 2006). Moreover, the results raise new possibilities regarding differential modulation of ionic currents in diverse tissues in the inner ear, but with a common theme of altering the electrical gain in hair cells.

We have demonstrated that sulfhydryl modification of L and non L-type channels in hair cells results in a reduction in whole cell Ca\(^{2+}\) currents, which is readily reversed by disulfide reduction. At the unitary current level, the decline in macro-
scopic current was mediated by a decrease in $P_o$ and open time, with no change in unitary channel conductance, in agreement with changes in gating but not permeation of $\text{Ca}^{2+}$ channels. However, prolonged exposure of NO to hair cells induces further inhibition of $\text{Ca}^{2+}$ currents by activation of a cGMP-signaling pathway. These inhibitory effects of NO and/or NO donors on $\text{Ca}^{2+}$ currents cannot be reversed by sulfhydryl-reducing agents after prolonged exposure, as would be expected for a direct modulation. In support of this assertion, we showed that the second component of inhibition of the whole cell $\text{Ca}^{2+}$ current was abolished by KT-5823, a protein kinase G inhibitor. Additionally, dibutyryl-cGMP produces a reduction of the $\text{Ca}^{2+}$ current and, in the presence of the cGMP analog, NO induces a single component of the current inhibition. Consistent with previous reports from hair cells in rat semicircular canals and cardiac cells (Almanza et al. 2007; Campbell et al. 1996), the data suggest that hair-cell $\text{Ca}^{2+}$ current can be modulated by both direct (S-nitrosylation/oxidation) and indirect (cGMP-dependent) pathways. The present experiments also demonstrate the presence of constitutive NOS in hair cells and reveal a NO-mediated cholinergic response that, until now, has not been realized in the auditory and vestibular settings. In contrast, it has been established that cholinergic modulation of heart rate is mediated by the release of NO in cardiac cells (Han et al. 1998). The $\text{Ca}^{2+}$ dependence of ACh action on release of NO is in keeping with the requirement for $\text{Ca}^{2+}$-mediated calmodulin activation of NOS (Chvanov et al. 2006; Song et al. 2008), in that the $\text{Ca}^{2+}$ chelators EGTA/BAPTA attenuate the ACh-mediated effects (Han et al. 1998). Moreover, it can be inferred from our data that the source of $\text{Ca}^{2+}$ may be derived in part from influx through hair-cell $\text{Ca}^{2+}$ channels.

For the large conductance potassium current, the actions of NO may involve direct nitrosylation of the channel since the reducing agent DTT reversed the inhibitory effects of NO on whole cell and single-channel currents. Similar to $\text{Ca}^{2+}$ channel activity, the direct effects of NO on the BK channels is derived from alterations in the $P_o$; findings that are akin to a previous report on NO-induced activation of BK channels in mesenteric and spiral modiolar arteries (Jiang et al. 2004; Mistry and Garland 1998). By increasing BK channel and reducing $\text{Ca}^{2+}$ channel activities, the net effect will promote membrane hyperpolarization and reduction in excitability in accord with the NO-mediated actions on rat vestibular hair cells (Almanza et al. 2007). However, the complexity of the actions of NO cannot be underestimated since it has an inhibitory effect on a large conductance voltage-activated K$^+$ current in type I vestibular hair cells, $I_{\text{K,L}}$ (Chen and Eatock 2000), which is expected to increase the electrical gain of hair cells. Thus the resultant effects of NO actions may depend on the composition and magnitude of ionic conductances relative to the total membrane conductance in a given hair cell.

NO is released as a second messenger that exerts many of its actions by way of several interrelated redox forms to produce direct and indirect features and reactivities on proteins. The by-products of NO consist of nitrogen (N)-oxides, which exhibit reactivity profiles that are distinct from NO itself (Ahmmed et al. 2001; Stamler et al. 1992). Indeed, 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 (Ruppersberg et al. 1991). A second level of NO-mediated effects is through an indirect pathway involving activation of adenyl cyclase and guanylyl cyclase and increased levels of cGMP (Ahmmed et al. 2001). Consequently, there is an ensuing activation of protein kinases and phosphorylation of ion channels, resulting in regulation of their functions. These NO-mediated effects have been found to govern a series of biological mechanisms including vasodilation, neurotransmission, and neuronal plasticity (Snyder 1992; Snyder and Bredt 1992). A series of reports have shown high levels of expression of NOS in the cochlea and vestibular end organs (Fessenden and Schacht 1998; Fessenden et al. 1994; Gosepath et al. 1997; Lysakowski and Singer 2000). Additionally, it has been reported that NO donors suppress cochlear potential and outer hair-cell responses (Chen et al. 1995). Using electrochemical and fluorescence detection methods of NO, Shi et al. (2002) measured increased levels of NO in the cochlea after noise exposure (Shi et al. 2002). Here, Bay K 8644 (5 μM) was included in the bath and the pipette. Plots of open-time distribution in the control (left), NO-modified (middle), and DTT-modified (right) L-type channels are shown. The solid lines represent bi- and triexponential fits to the data, with time constants indicated on the plots. Compared with control, time constants obtained from NO-modified patches were significantly abbreviated by exposure to 100 nM NO [e.g., 2.2 ± 0.01 ms (control) vs. 0.50 ± 0.02 ms (n = 4; P < 0.05) in NO-modified patches, respectively]. Note that DTT (0.2 mM) restored the fast open-time constant (1.1 ± 1.5; n = 3; P = 0.7). Also important, the fast open-time constant was eliminated in NO-modified L-type single channel current kinetics.
et al. 2002). However, what is most unclear is the physiological significance of NO release.

The efferent nerve system, projecting to the inner ear or its homologue, from the olivocochlear pathway exists in a wide range of organisms, raising the possibility that evolutionary preservation of the system is of vital importance to inner ear functions (Dilly 1976; Guinan Jr et al. 1983). For example, ACh is the neurotransmitter released by the medial efferent fibers and analogous synaptic feedback has been identified in hair cells of the fish lateral line, amphibian, and avian inner ear (Eybalin 1993). ACh released from efferent nerve terminals produces fast hyperpolarization of hair cells by activating Ca\(^{2+}\)/H11001-dependent small conductance (SK) K\(^+\) channels (Elgoyhen et al. 2001; Housley et al. 2006). Moreover, repetitive shock trains of efferent fibers produce a second slow hyperpolarization, but the underlying mechanism remains unaccountable (Sridhar et al. 1997). The unique expression and tissue specificity in efferent terminals and hair cells (Fessenden and Schacht 1998; Lysakowski and Singer 2000) indicate that NOS might serve specialized physiological functions. Indeed, it has been suggested that NO release either from hair cells or efferent nerve terminals might mediate negative feedback mechanisms on hair cells (Fessenden and Schacht 1998). Moreover, the effects of NO on hair cells cannot be generalized since it appears to have opposing actions on other hair-cell types (Chen and Eatock 2000). NO-mediated activation of cyclic nucleotides and its relevance efferent system modulation of hair cells and cochlear amplification dovetail well with previous studies (Dek et al. 2005; Szönyi et al. 1999). Overall, the present findings contribute to the growing awareness that actions of NO in the operational status of hair cells may provide an essential mechanism for custom modulation of hair-cell functions.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


