Release and Elementary Mechanisms of Nitric Oxide in Hair Cells

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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. 2000). To maintain its acute sensitivity and to protect itself from mechanical stimuli, making them ideally prone to mechanical reactions, stimulation of hair cells is coupled directly to the stimulus, and thereby generating the characteristic membrane potential oscillations. This resulted in a rightward shift in the frequency–current relationship and altered the excitability of hair cells. Our data suggest that these effects ensue because NO reduces whole cell Ca2+ current and drastically decreases the open probability of single-channel events of the L-type and non-L-type Ca2+ channels in hair cells, an effect that is mediated through direct nitrosylation of the channel and activation of protein kinase G. Finally, NO increases the magnitude of Ca2+-activated K+ currents via direct NO nitrosylation. We conclude that NO-mediated inhibition serves as a component of efferent nerve modulation of hair cells.

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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\(^{2+}\) frog Ringer solution (in 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 placed in a recording chamber containing 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 saccula were disrupted by exposing the perilymphatic surface to 4 mM ethylene glycol-bis-(\(\beta\)-aminoethyl ether)-N,N,N',N'-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 then collected. Frogs were killed and inner ears were quickly removed by 10.220.33.1 on November 2, 2016 http://jn.physiology.org/ Downloaded 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 Fick equation. The product of a theoretical model developed from the Fick equation. D: data collected from a frog saccule. The excursion distance of the microsensor was 5 µm away from the saccule. The mean NO flux was 115 ± 20 nmol·m$^{-2}$·s$^{-1}$ (n = 15), compared with background reading $1 \pm 8$ nmol·m$^{-2}$·s$^{-1}$ (n = 15). E: representative recording from a saccule (dotted line) and on application of 5 µM acetylcholine (ACh). Superimposed on the same plot (solid line) is a depiction of the inhibitory effects of $N^\omega$-nitro-$l$-arginine methyl ester (L-NAME).

**FIG. 1.** Measurement of nitric oxide (NO) efflux. A: schematic diagram of the NO-sensitive microsensor. The microsensor was constructed by inserting 5-µm carbon fiber and then heating and pulling the glass electrode. Epoxy was backfilled into the electrode to seal the fiber with the glass electrode. A graphite paste was used to cement a copper wire to the carbon fiber. The tip of the electrode was beveled, coated with Nafion, and plated with o-phenylenediamine. B: the electrodes were calibrated with 21 standards with known concentrations of NO and the selectivity was tested against ascorbic acid. The gradient of the linear regression of the calibration line was used to calculate the flux of NO. C: NO flux in relation to the distance from a source pipette (diameter ~3 µm). Symbols represent measured values and the solid squares (●) represent the product of a theoretical model developed from the Fick equation. D: data collected from a frog saccule. The excursion distance of the microsensor was 5 µm at a frequency of 0.3 Hz. When in the “epithelium” position the electrode was about 2 µm from the tissue. Background represents data collected at a position 2,000 µm away from the saccule. The mean NO flux was 115 ± 20 nmol·m$^{-2}$·s$^{-1}$ (n = 15), compared with background reading $1 \pm 8$ nmol·m$^{-2}$·s$^{-1}$ (n = 15). E: representative recording from a saccule (dotted line) and on application of 5 µM acetylcholine (ACh). Superimposed on the same plot (solid line) is a depiction of the inhibitory effects of $N^\omega$-nitro-$l$-arginine methyl ester (L-NAME). F: summary data on the effects of $N^\omega$-nitro-$d$-arginine methyl ester (d-NAME; n = 7), L-NAME (n = 8), ACh (n = 9), Ca$^{2+}$-free solution (n = 5), and Ca$^{2+}$-free + ACh (n = 7) on NO efflux.

**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 filled with a lasser 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). $N^\omega$-Methyl-$d$-glucamine (NMG) was used to substitute for divalent Ca$^{2+}$ and to maintain an osmolarity of about 280 mmosmol. 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, 1 CaCl$_2$, 5 4-AP, and 5 HEPES pH 7.4 with TEA-OH, to shift the resting potential to about 0 mV (Rodriguez-Contreras and Yamoah 2003). For Ca$^{2+}$-activated K$^+$ channel currents, the bath solution contained (in mM) 110 KCl, 2 CaCl$_2$, 5 4-AP, and 5 HEPES 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-Contreras et al. 2002). All experiments were carried out at room temperature (~21°C).

**Chemicals and solutions**

Several chemicals were used. These include $N^\omega$-nitro-$l$-arginine methyl ester and $N^\omega$-nitro-$d$-arginine methyl ester (l-NAME and
d-NAGM, respectively; Sigma), diithiothreitol (DTT), a thiol reducing reagent, sodium nitroprusside (SNP, Sigma), 3-morpholinosydnonimine (SIN-1, Molecular Probes, Eugene, OR), and 5-nitroso-N-acetylpenicillamine (SNAP, Molecular Probes), NO donors. All other chemicals were obtained from Sigma, unless otherwise noted.

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.

RESULTS

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 l-NAGM 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, l-NAGM inhibited the NO efflux to 2 ± 12 nmol·m⁻²·s⁻¹ (n = 8). In contrast, application of the inert enantiomer (d-NAGM) 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 l-NAGM, 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⁺. Replacement of the normal frog saline and application of a Ca²⁺-free saline, which included the Ca²⁺ chelator EGTA (5 mM), decreased the baseline NO efflux and attenuated the ACh-induced rise in NO efflux.

NO and NO donors decrease hair-cell membrane excitability

The functional consequences of the release of NO in or at the vicinity of hair-cell membrane properties were examined at the current-clamp recording conditions, using perforated (amphoterin) patches. As illustrated in Fig. 2A (top left) isolated hair cells in the frog saccule typically exhibit baseline membrane oscillations. Of 56 cells that were sampled, 50 (89%) had spontaneous baseline membrane oscillations, whereas the remaining 6 (11%) were quiescent. Injection of current resulted in increased membrane fluctuations until the characteristic frequency oscillation ensued. Figure 2A shows examples of such membrane voltage oscillations. In contrast to recordings from control hair cells, application of SNAP not only attenuated the baseline membrane oscillations, but it also resulted in increased magnitude of the injected current required to generate characteristic membrane potential oscillations (Fig. 2B). The resulting effects of NO were to shift the frequency–current relationship to the right, thus altering the excitability of hair cells (Fig. 2C). Similar effects were observed with the NO donor SIN (10 μM) and by direct application of NO (100 nM) solution. Within 3 min of application of NO donors/NO solution, the effect was reversed readily by washing the drug with normal frog saline containing about 200 μM of the reducing agent DTT. DDT (200 μM) alone had no effect on membrane potential oscillations (data not shown); however, sustained exposure of hair cells to NO donors/NO (>7 min) resulted in only partial recovery after washout with DTT.

The mechanisms of membrane oscillation in hair cells in lower vertebrates have been ascribed mainly to the interplay between activation of voltage-gated Ca²⁺ currents (VGCCs) and Ca²⁺-activated K⁺ currents (BKs), although the contribution of other voltage-gated K⁺ currents in hair cells that are tuned to low frequencies have been reported (Armstrong and Roberts 1998; Art et al. 1993; Fuchs 1992; Fuchs and Evans 1988; Holt et al. 1997; Hudspeth and Lewis 1988b). To determine the underlying mechanisms for the NO-induced changes in hair-cell excitability, we studied the effects of the NO donors SNAP, SNP, and NO on VGCC and BK currents. To record Ca²⁺ currents, outward K⁺ currents were blocked with external TEA, 4-AP, and internal Cs⁺. Figure 3, A–C shows representative traces of Ca²⁺ currents and the effects of NO, dibutylr-3,5′-cyclic guanosine monophosphate (cGMP),
presence of NO donors, the whole cell Ca\textsuperscript{2+} membrane gain of hair cells.

Aside from the reduction of the magnitude of the Ca\textsuperscript{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 Ca\textsuperscript{2+} currents could reduce the excitability of hair cells and increase the threshold for the induction of membrane oscillations, enhancement of outward K\textsuperscript{+} currents could produce similar outcomes through membrane repolarization. We recorded whole cell K\textsuperscript{+} currents and determined that only the Ca\textsuperscript{2+}-dependent component was affected by NO (data not shown). We examined the effects of NO on the Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) current by blocking the voltage-gated K\textsuperscript{+} 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 (ΔV = 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 Ca\textsuperscript{2+} current. However, the increase in magnitude of the outward K\textsuperscript{+} current was larger than could be ascribed to a reduction of NO-induced block of the Ca\textsuperscript{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 Ca\textsuperscript{2+} and BK currents

Hair cells in the frog saccule express multiple Ca\textsuperscript{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 Ca\textsuperscript{2+} currents obtained from a cell-attached patch containing a nimodipine-insensitive channel. The patch pipette contained 65 mM Ca\textsuperscript{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 Ca\textsuperscript{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\textsubscript{o}) was robust and reversible by DTT (0.2 mM) (mean P\textsubscript{o} values at test potential of −50 mV were: control, 0.18 ± 0.07; after application of NO, 0.07 ± 0.01; and after DTT, 0.20 ± 0.05; n = 9). Similar results were obtained when SNAP (100 μM) was used (mean P\textsubscript{o} values at test potential of −50 mV were: control, 0.21 ± 0.05; after application of SNAP, 0.09 ± 0.03; and after DTT, 0.19 ± 0.03; n = 5).

FIG. 2. NO donor S-nitroso-N-acetylpenicillamine (SNAP) decreases the membrane gain of hair cells. A: a family of oscillations of hair-cell membrane potentials in the absence and in response to injection of current steps (indicated) for controls (left) and after application of 10 μM SNAP. The NO donor reduces baseline membrane oscillations. B: the resulting effects of the NO donor is a shift in the frequency–current (F–I) relationship to the right. C: the frequency of membrane potential oscillations as a function of injected current for a hair cell. The oscillations at current “on” in solid symbols and oscillations at current “off” are in open symbols. The solid curves were calculated using the equation (Crawford and Fettiplace 1981): \( F (I) = F_o \log \left[ 1 + \mu (I - I_o) \right] \), where \( F_o \) is the frequency of oscillation at a given current step of amplitude \( I \), \( I_o \) is the minimum outward current required to suppress the oscillations, and \( F \) and \( \mu \) are constants for the cell with units of Hz and reciprocal current, respectively. For the example shown, the NO donor appears to shift the characteristic frequency of the cell from about 55 to 40 Hz.

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 Ca\textsuperscript{2+} current was reduced by 34.0 ± 6.0% by SNAP (n = 11), 31.2 ± 4.1% (n = 9) by SNP, and 35.8 ± 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 curvature (Fig. 3E). The effect of NO on the probability of the non L-type channel openings (P\textsubscript{o}) was robust and reversible by DTT (0.2 mM) (mean P\textsubscript{o} values at test potential of −50 mV were: control, 0.18 ± 0.07; after application of NO, 0.07 ± 0.01; and after DTT, 0.20 ± 0.05; n = 9). Similar results were obtained when SNAP (100 μM) was used (mean P\textsubscript{o} values at test potential of −50 mV were: control, 0.21 ± 0.05; after application of SNAP, 0.09 ± 0.03; and after DTT, 0.19 ± 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 μM). Examples of the L-type Ca$^{2+}$ channel currents traces recorded from a holding potential of −70 mV and step potential of −30 mV are shown in Fig. 6A–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 $K_o$) suggests 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 μM 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 p S (n = 7: Fig. 9D). In contrast to the effects of NO on the Ca2+/H11001 channels, application of NO to the BK channel resulted in a substantial increase in P0 of the channel (Fig. 9B). The diary plot of the P0 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+/H11001-dependent manner. The electrophysiological consequences of the release of NO are a

FIG. 4. Whole cell recordings of Ca2+/H11001-activated K+/H11001 current in hair cells. The amplitude of outward Ca2+/H11001-activated K+/H11001 currents was increased by NO. A: outward K+/H11001 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-aminopyridine (4-AP) and tetraethylammonium (TEA) to suppress other outward K+ currents (see METHODS). The current was enhanced in response to increased external Ca2+/H11001 (data not shown). The Ca2+/H11001-activated K+/H11001 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+/H11001-activated K+/H11001 current, hair cells were held at −70 mV and stepped briefly (25 ms) to −30 mV to activate the inward Ca2+/H11001 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+/H11001 current.

FIG. 5. Single-channel Ca2+/H11001 current fluctuations in hair cells plummeted in NO but the effect was reversed by DTT. Representative single-channel traces recorded using 65 mM Ca2+/H11001 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+/H11001 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 Ca$^{2+}$ channels. However, prolonged exposure of NO to hair cells induces further inhibition of Ca$^{2+}$ currents by activation of a cGMP-signaling pathway. These inhibitory effects of NO and/or NO donors on 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 Ca$^{2+}$ current was abolished by KT-5823, a protein kinase G inhibitor. Additionally, dibutyryl-cGMP produces a reduction of the 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 Ca$^{2+}$ current can be modulated by both direct (S-nitrosoylation/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 Ca$^{2+}$ dependence of ACh action on release of NO is in keeping with the requirement for Ca$^{2+}$-mediated calmodulin activation of NOS (Chvano et al. 2006; Song et al. 2008), in that the 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 Ca$^{2+}$ may be derived in part from influx through hair-cell 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 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 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, $I_{K,L}$ (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 distinct 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 adenyllyl 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). 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 $\text{Ca}^{2+}$-dependent small conductance (SK) $\text{K}^{+}$ 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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