Modulation of Voltage-Gated Ca\textsuperscript{2+} Current in Vestibular Hair Cells by Nitric Oxide

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Almanza A, Navarrete F, Vega R, Soto E. Modulation of voltage-gated Ca\textsuperscript{2+} current in vestibular hair cells by nitric oxide. J Neurophysiol 97: 1188–1195, 2007. First published December 20, 2006; doi:10.1152/jn.00849.2006. The structural elements of the nitric oxide–cyclic guanosine monophosphate (NO–cGMP) signaling pathway have been described in the vestibular peripheral system. However, the functions of NO in the vestibular endorgans are still not clear. We evaluated the action of NO on the Ca\textsuperscript{2+} current in rat vestibular hair cells isolated from the semicircular canal crista ampullaris of the rat (P14–P18) by using the whole cell and perforated-cell patch-clamp technique. The NO donors 3-morpholinosydnonimine (SIN-1), sodium nitroprusside (SNP), and (±)-(E)-4-ethyl-2-[(Z)-hydroxymino]-5-nitro-3-hexen-1-yl-nicotinamide (NOR-4) inhibited the Ca\textsuperscript{2+} current in hair cells in a voltage-independent manner. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) prevented the inhibitory effect of SNP on the Ca\textsuperscript{2+} current. The selective inhibitor of the soluble form of the enzyme guanylate cyclase (sGC), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), also decreased the SNP-induced inhibition of the Ca\textsuperscript{2+} current. The membrane-permeant cGMP analogue 8-Br-cGMP mimicked the SNP effect. KT-5823, a specific inhibitor of cGMP-dependent protein kinase (PGK), prevented the inhibition of the Ca\textsuperscript{2+} current by SNP and 8-Br-cGMP. In the presence of N-ethylmaleimide (NEM), a sulfhydryl alkylating agent that prevents the S-nitrosylation reaction, the SNP effect on the Ca\textsuperscript{2+} current was significantly diminished. These results demonstrated that NO inhibits in a voltage-independent manner the voltage-activated Ca\textsuperscript{2+} current in rat vestibular hair cells by the activation of a cGMP-signaling pathway and through a direct action on the channel protein by a S-nitrosylation reaction. The inhibition of the Ca\textsuperscript{2+} current by NO may contribute to the regulation of the intracellular Ca\textsuperscript{2+} concentration and hair-cell synaptic transmission.

I N T R O D U C T I O N

Nitric oxide (NO) has emerged as an important signaling molecule with a functional role in both central and peripheral systems. Experimental evidence indicates that once synthesized, NO rapidly diffuses into neighboring cells, thus regulating their activity. The modulation of ionic channels has been suggested as one of the mechanisms mediating the physiological functions of NO, including relaxation of vascular smooth muscle (Blatter and Wier 1994; Lewis et al. 2005), neurotransmission, synaptic plasticity, and neurodegenerative processes (Garthwaite and Boulton 1995; Rand and Li 1995; Schuman and Madison 1994). Ionic channel modulation by NO can be produced by S-nitrosylation of target proteins (Ahern et al. 2002; Jaffrey et al. 2001) or indirectly through second messengers involving the activation of sGC (the soluble form of the enzyme guanylate cyclase) and increased levels of cyclic guanosine monophosphate (cGMP) (Bredt and Snyder 1989; Garthwaite and Boulton 1995).

In the acoustico-lateral system there is growing evidence of the presence of a NO–cGMP signaling pathway. There is evidence that neuronal- and endothelial nitric oxide synthases (NOS) and enzymatic activities of sGC, NOS, and cGMP-dependent protein kinase I (PKG-I) are co-localized in supporting cells of the organ of Corti (Fessenden and Schacht 1997; Fessenden et al. 1994; Gosepath et al. 1997; Tian et al. 1999; Zdanski et al. 1994). Activity of the enzyme NADPH-diaphorase (NADPH-d), which is now known to be NOS, was demonstrated in the electroreceptors of a gymnotiform teleost Apteronotus leptorhynchus (Turner and Moroz 1995) and in the neuromasts of the axolotl Ambystoma tigrinum (Vega et al. 2006). These sensory receptors are evolutionary related to the hair cells of the vertebrate inner ear. In the vestibular system of the chinchilla and rat, NADPH-d histochemistry and NOS-I positive immunoreactivity were found in a subpopulation of vestibular-afferent neurons contacting hair cells (Lysakowski and Singer 2000). NOS-I, calretinin, and sGC were co-localized in calyx-ending afferent neurons of the chinchilla and both sGC and calmodulin, an important cofactor of NOS (Bredt 1999), were co-localized in type I and type II hair cells (Desai et al. 2004; Dhaliwal and Lysakowski 2000). NOS was co-localized with sGC and cGMP in the sensory epithelia and the vestibular nerve fibers of the guinea pig and mice (Hess et al. 1998a,b). These results indicate that the elements of the NO–cGMP pathway are expressed in hair cells and efferent and afferent neurons. Functionally, it has been shown that NOS inhibitors diminished the afferent resting discharge in the amphibian vestibular and lateral line systems (Flores et al. 1996, 2001) and had inhibitory–excitatory effects in the cephlopod statocyst (Tu and Bodelman 1999, 2000). The production of NO was previously reported in guinea pig vestibular hair cells, which increased after stimulation by L-arginine (Takumida and Anniko 2000).

However, there are only a few reports in the literature, as far as we were able to determine, concerning the action mechanism of NO at the cellular level in the acoustico-lateral system. It was shown that cGMP and NO donors inhibit the low-voltage–activated potassium conductance ($I_{K,L}$) specific for type I hair cells, thus increasing the hair cells gain and functioning as a positive feedback mechanism capable of boosting afferent neurotransmitter release (Behrend et al. 1997; Chen and Eatock 2000). In the guinea pig cochlea nitroprusside...
reduced endocochlear potential, sound-evoked potentials, electromotility, and outward current from isolated outer hair cells (Chen et al. 1995).

Rat vestibular hair cells express L-type Ca²⁺ channels formed by the α₁D subunit (Almanza et al. 2003; Bao et al. 2003). Given the evidence of the effects of NO–cGMP on Ca²⁺ channels in different cell types and considering a preliminary report that suggests that NO reduced the open probability of Ca²⁺ channels in bullfrog hair cells (Rodriguez-Contreras et al. 2000), it seems feasible that NO might modulate Ca²⁺ channels and constitute an effective mechanism for the control of the intracellular Ca²⁺ level in hair cells and afferent transmitter release. To evaluate this possibility we studied the action of NO on Ca²⁺ channels in hair cells. Our results show that NO inhibits the voltage-activated Ca²⁺ current in rat vestibular hair cells by the activation of a cGMP-signaling pathway and through a direct action on the channel protein by the S-nitrosylation reaction.

METHODS

Experiments were made on young Long–Evans rats (P14–P18), supplied by Claude Bernard animal house of the Autonomous University of Puebla. Animal care and procedures were in accordance with the Reglamento de la Ley General de Salud en Materia de Investigación para la Salud de la Secretaría de Salud de México. All efforts were made to minimize animal suffering and to reduce the number of animals used, as outlined in the Guide for the Care and Use of Laboratory Animals issued by the National Academy of Science.

Cell preparation

Hair cells were enzymatically dissociated from the semicircular canal crista ampullaris of the rat as reported previously (Almanza et al. 2003). In brief, tissue pieces containing the vestibular sensory epithelia were incubated in normal Tyrode solution (see Solutions) containing 0.2 mg/mL IA type collagenase for 7 min at 35°C, followed by cell incubation for 10 min at 35°C in a Ca²⁺- and Mg²⁺-free Tyrode solution with 1 mg/mL porcine-trypsin. Finally, tissue was washed for 10 min at 4°C in a Ca²⁺- and Mg²⁺-free Tyrode solution containing 1 mg/mL serum bovine albumin. All enzymes were from Sigma–Aldrich (StLouis, MO). The tissue was then placed in the recording chamber on an inverted microscope stage (Nikon Diaphot, Tokyo) and cells were isolated by gently triturating the tissue with fire-polished Pasteur pipettes. Cells settled and adhered to the bottom of the recording chamber within 10 min. The recording chamber was continuously perfused with the extracellular solution designed for the Ca²⁺ current recording (see Solutions) at a constant rate (0.5 mL/min) using a peristaltic pump (Microperpex, LKB, Sweden).

Cell identification

Isolated cells were visualized with phase-contrast optics. Type I and type II hair cells were identified using morphological criteria. Previous studies have shown that in hair cells there is a good relation between their morphology and electrophysiological properties, hence cellular types can be distinguished morphologically (Almanza et al. 2003; Chen and Eatock 2000; Rüsch et al. 1998). Cells that exhibited a flask-shaped body with a highly refringent cuticular plate wider than the hair cell neck were considered to be type I hair cells and cells exhibiting cylindrical shapes and cuticular plate hardly distinguishable under phase contrast optics were considered to be type II hair cells.

Records

All experiments were done at room temperature (20–22°C). Whole cell recordings were done with the patch-clamp technique according to the method described by Sakmann and Neher (1984). Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) using a Flaming-Brown electrode puller (P80/PC; Sutter Instruments, San Rafael, CA) and had an open tip resistance of 3–5 MΩ. Whole cell current recordings were made with an axopatch 200B amplifier (Molecular Devices, Union City, CA). Signals were low-pass filtered at 2 kHz and digitized at 5 kHz. Command pulse generation and data sampling were controlled by pClamp 8.0 software (Molecular Devices) using a 12-bit AD/DA converter (Digidata 1200, Molecular Devices). Both the membrane capacitance (Cm) and 80% of the series resistant (Rs) were electronically compensated. The average Cm was 7 ± 1 pF and the average Rs was 17 ± 1.8 Ω (n = 72). The maximum voltage errors caused by uncompensated Rs were ≤1 mV, corresponding to currents ≤100 pA, therefore no voltage correction for uncompensated Rs was needed. The junction potential was calculated with the generalized Henderson liquid-junction potential equation (Barry 1994) using pClamp 8.0 software (Molecular Devices). Voltages were corrected for a 7.9 mV liquid-junction potential for the Ba²⁺ extracellular solution and Cs⁺ and TEA intracellular Ca²⁺ solutions (see Solutions). Some experiments were made with permeabilized patch techniques. For these the pipette solution containing 200 μM nystatin was sonicated, then back filled into the recording pipette.

Solutions

The normal Tyrode solution was (in mM) 140 NaCl, 5.4 KCl, 1.2 MgCl₂, 3.6 CaCl₂, 10 HEPES, and 10 Glucose. The Ca²⁺- and Mg²⁺-free Tyrode solution was (in mM) 140 NaCl, 5.4 KCl, 10 HEPES, 10 Glucose, and 1 EGTA. For Ca²⁺ current recording, extracellular Ba²⁺ was used as a current carrier and intracellular Cs⁺-TEA solutions were used to block K⁺ current. The extracellular solution with Ba²⁺ was (in mM) 23 TEACl, 30 CsCl, 55 NaCl, 0.1 CaCl₂, 20 BaCl₂, 10 HEPES, and 10 Glucose. The patch electrodes were filled with a solution containing either (in mM) 23 TEACl, 69 CsCl, 0.1 CaCl₂, 10 HEPES, 80 NMDG⁺, 10 EGTA, 0.1 GTPNa, and 2 ATPMg for whole cell patch experiments or 138 CsCl, 0.1 CaCl₂, 10 HEPES, and 10 EGTA for the perforated-patch mode. The pH of all external solutions was adjusted to 7.4 with NaOH. The pH of intracellular solutions were adjusted to 7.2 with HCl or CsOH. The osmolarity for all solutions was adjusted to 300 mOsm.

Drugs

The compounds used as NO donors were 3-morpholinosydnonimine (SIN-1; Molecular Probes, Eugene, OR), sodium nitroprusside (SNP; RBI, Natick, MA), (±)-(E)-4-ethyl-2-[(Z)-hydroxymethyl]-5-nitro-3-hexen-1-yl-nicotinamide (NOD; Sigma–Aldrich, St Louis). The standard light beam of the microscope was directed onto the investigated cell to favor the NO production. The compound 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (CPTIO; Molecular Probes) was used as a nitric oxide scavenger. To study the participation of the cGMP-PKG pathway, we used 8-Br-cGMP (Sigma–Aldrich) as a membrane-permeant cGMP analog. An inhibitor of sGC, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma–Aldrich) and a specific inhibitor of PKG, KT-5823 (Sigma–Aldrich) were also used. N-ethylmaleimide (NEM; Sigma–Aldrich), which prevents S-nitrosylation of proteins, was used to study the participation of a cGMP-independent mechanism. Because SIN-1, SNP, 8-Br-cGMP, and NEM are light sensitive, experiments with them were done in a darkened room.

The compounds SNP, SIN-1, NOD-4, CPTIO, and 8-Br-cGMP were applied by microperfusion with a constant flow of 10 μL/min.
For this, after the last control recording, a Teflon microtube was drawn close to the cell being recorded and the desired solution was applied by pressure ejection from an electronic microsyringe (Baby Bee, BAS, Lafayette, IN). The recording chamber was designed to have a uniform solution flow from the inflow to the outflow side. The cells selected for the experiment were recorded sequentially starting from the outflow side of the chamber, thus ensuring that previous perfusion of drugs onto a cell did not affect successive recordings. No cell was exposed to more than one drug except for those experiments designed to study drug interactions. In the experiments done to study sGC inhibition, the ODQ was perfused into the bath for 25 min before the recording to allow it to diffuse into the cells. The KT-5823 and NEM were applied intracellularly directly by the recording pipette.

Data analysis

Recordings were analyzed off-line with pClamp 8.0, SigmaPlot 8.0 (Systat Software, Richmond, CA), and Origin 6.0 software (Microcal Software, Northampton, MA). For the curve-fitting routines a non-linear least-squares method was used.

To construct the current–voltage relationships (I–V), the Ca\(^{2+}\) currents were elicited by 300 ms voltage steps between −100 and +50 mV from a holding potential of −60 mV with 10 mV increments every 5 s. The Ca\(^{2+}\) current amplitude was measured between 155 and 175 ms after the start of voltage steps. The current produced between −100 and −70 mV was fitted with a linear function that was subtracted assuming a linear leak throughout the whole I–V relationship (Martinez-Dunst et al. 1997).

A single Boltzmann function was fitted to the I–V relationship between −60 and −10 mV, which corresponds to the growth region of the Ca\(^{2+}\) conductance (Bao et al. 2003). The function used was

$$I(V) = \frac{I_{\text{max}}}{1 + \exp\left(\frac{V_{1/2} - V}{S}\right)}$$

where \(I_{\text{max}}\) is the maximum current, \(V\) is the voltage, \(V_{1/2}\) is the voltage at half channel activation, and \(S\) is the slope of the curve at this midpoint.

The effect of different drugs on the Ca\(^{2+}\) current was evaluated by using 300 ms depolarizing test pulses to −10 mV from a holding potential of −60 mV every 4 s. The amplitude of the Ca\(^{2+}\) current was measured after ≈2 min of drug perfusion.

Statistics

Numerical data are presented as the mean ± SE for at least four measurements, unless otherwise stated. Statistical differences were determined using a Student's t-test. \(P < 0.05\) was used to denote statistical difference between the groups.

Results

Under control conditions, using the Ba\(^{2+}\) extracellular solution, depolarizing voltage steps of 300 ms from a holding potential of −60 mV caused an inward current that activated above −60 mV, reached its maximum at −18 mV (\(n = 36\)), and showed fast activation kinetics and no significant inactivation. The \(V_{1/2}\) for the control Ca\(^{2+}\) current was −37 ± 0.5 mV and the \(S\) was 5 ± 0.1 mV. This current was identified as a Ba\(^{2+}\) current entering the cell through voltage-dependent Ca\(^{2+}\) channels and thus will be referred to as \(I_{\text{Ca}}\). These characteristics are in agreement with previous results in the rat and chick, indicating that the \(I_{\text{Ca}}\) in type I and type II hair cells is a L-type Ca\(^{2+}\) current (Almanza et al. 2003; Bao et al. 2003; Masetto et al. 2005). A similar \(I_{\text{Ca}}\) was found when recording with the perforated patch-clamp technique (\(n = 6\); data not shown).

Modulation of the Ca\(^{2+}\) current by nitric oxide donors

To evaluate the effects of NO on the \(I_{\text{Ca}}\) in hair cells, SIN-1, NOR-4, and SNP that release NO were used. Initially we studied the action of SNP in hair cells identified as type I or type II. The application of 100 \(\mu\)M SNP, an inorganic nitroso compound, decreased the \(I_{\text{Ca}}\) in type I hair cells (53 ± 5%; \(n = 5\), \(P = 0.0007\)) (Fig. 1) with no significant effect on the \(I_{\text{Ca}}\) activation or inactivation rates (Fig. 1A, inset). The effect of SNP reached its maximum after about 3 min of its perfusion.
(Fig. 1B) and was partially reversible, recovering to 75% of the control current after 2 min of drug washout. The $V_{1/2}$ was shifted to the right by 2.5 ± 1 mV (100 μM SNP; $P = 0.07$) (Fig. 1C). In cells identified as type II hair cells the application of 100 μM SNP also decreased the $I_{Ca}$ (49.7 ± 8%; $n = 7, P = 0.002$) with no significant effect on the $I_{Ca}$ activation or inactivation rates (data not shown). No significant difference was found between the $I_{Ca}$ in type I or type II hair cells nor did the action of SNP show any significant difference ($P = 0.7$); thus no further distinction of the cell type was done. To avoid dialysis of cytoplasmic elements, such as sGC, during the whole cell recording, experiments using the perforated patch-clamp technique were also made. In this condition, the application of 100 μM SNP decreased the $I_{Ca}$ in a fashion similar to that found with the whole cell recording (59 ± 8%; $n = 6$ as compared with 53 ± 5%; $n = 5, P = 0.67$) (data not shown). Because no significant difference was found between whole cell and perforated recordings, the rest of the experiments were done using the whole cell patch clamp.

Because SNP at nanomolar concentrations was previously described as being able to produce an enhancement of the L-type Ca$^{2+}$ current in frog myocytes (Méry et al. 1993), we investigated the action of SNP at lower concentrations (Fig. 1D). SNP, applied at both 1 μM and 100 nM, significantly reduced $I_{Ca}$ (23 ± 7%; $n = 5, P = 0.03; 20 ± 4%; n = 8, P = 0.001$). After 2-min washout of the drug a recovery of roughly 80% of the control current was reached (see e.g., Fig. 1B). A concentration of 1 nM SNP produced a slight nonsignificant completely reversible decrease of the $I_{Ca}$ (9 ± 5%; $n = 4, P = 0.16$). No enhancement of the current was observed in any of the experiments.

Application of 1 μM SIN-1 significantly decreased the $I_{Ca}$ (45 ± 5%; $n = 4, P = 0.004$) (data not shown), with the maximum effect reached 3 min after the SIN-1 application. Washout of the drug produced a partial 60% recovery of the $I_{Ca}$ after 2 min. The SIN-1 (1 μM) did not significantly modify the $V_{1/2}$ or the $S$. Application of 100 μM SIN-1 diminished the $I_{Ca}$ (60 ± 9%; $n = 9, P = 0.0001$) (data not shown) and produced a significant positive shift of $V_{1/2}$ from −39 ± 0.4 to −35 ± 1.5 mV ($n = 9; P = 0.02$). No significant change of the slope was observed (S values of 5.4 ± 0.3 and 5.8 ± 0.3 mV; $P = 0.3$). The action of 100 μM SIN-1 was irreversible after a 2-min washout of the drug (data not shown).

A nonthiol-based NO donor NOR-4 (Kita et al. 1994) in concentrations of 100 (Fig. 2, A and B) and 1 μM (not shown) significantly decreased the $I_{Ca}$ (67 ± 8%; $n = 3, P = 0.0005$ and 38 ± 2%; $n = 4, P = 0.01$). For both concentrations the NOR-4 effect was irreversible (Fig. 2A). NOR-4 produced a nonsignificant displacement to the right of $V_{1/2}$ for $I_{Ca}$ (Fig. 2C). The above results indicate that the inhibitory action of the three NO donors tested is mediated by the common product of these drugs, nitric oxide. Because SNP also releases cyanide ions that may inhibit Ca$^{2+}$ currents (Biscoe and Duchen 1989), we decided to study the action of SNP concurrently with NO scavenger. The compound CPTIO (300 μM) produced a slight nonsignificant decrease of the $I_{Ca}$ 1 min after its perfusion (11 ± 3%; $n = 6, P = 0.07$) (Fig. 3, A and B). In the presence of CPTIO the inhibitory effect of 100 μM SNP on the $I_{Ca}$ was significantly reduced (12 ± 5%; $n = 6$, compared with 53 ± 5% caused by SNP alone, $P = 0.002$) (Fig. 3D). These results indicate that the inhibition of $I_{Ca}$ by SNP is caused by the production of NO and not by other products of SNP breakdown.

**NO activation of sGC modulates the Ca$^{2+}$ current**

To investigate whether the inhibition of the $I_{Ca}$ produced by NO is mediated by the activation of sGC and the subsequent increase in the levels of cGMP, the cells were treated for 25 min with 10 μM ODQ, a selective sGC inhibitor (Garthwaite et al. 1995). Subsequent coapplication of 100 μM SNP decreased the $I_{Ca}$ (11 ± 11%; $n = 9, P = 0.02$ compared with the inhibition by SNP alone), indicating that the action of SNP implies NO production and subsequent sGC activation (Fig. 3, C and D).

To further characterize the NO–cGMP pathway we investigated the action of the membrane-permeant cGMP analogue 8-Br-cGMP and of a specific inhibitor of the PKG, KT-5823. The 8-Br-cGMP in concentrations of 200 and 400 μM decreased the $I_{Ca}$ after 3 to 4 min of its application (29 ± 7%; $n = 4, P = 0.03$ and 50 ± 7%; $n = 4, P = 0.006$), thus mimicking the action of SNP (Fig. 4, A and B). The washout of the drug produced no significant recovery of the $I_{Ca}$ during the 2 to 3 min after drug removal (in only two of eight cells did the washout produce a partial recovery of the current magnitude). Boltzmann fitting showed that the $V_{1/2}$ of current activation shifts by 2 ± 2 mV (200 μM 8-Br-cGMP; $P = 0.03$) and 6 ± 3 mV (400 μM 8-Br-cGMP; $P = 0.01$) (Fig. 4C). Changes in the $S$ parameter were not statistically significant. In the presence of 10 μM KT-5823 the inhibitory effect of 400 μM 8-Br-cGMP on the $I_{Ca}$ was significantly reduced (31 ± 1%; $n = 7, P = 0.02$) (data not shown), indicating that the
inhibition of the $I_{\text{Ca}}$ produced by 8-Br-cGMP is mediated by the activation of the PKG. The KT-5823 (10 μM) also significantly inhibited the effect of 100 μM SNP (31.5 ± 3.6%; $n = 4$, $P = 0.007$ compared with the 53% inhibition by 100 μM SNP), indicating that the $Ca^{2+}$ channel modulation by NO also implies the participation of the NO–cGMP–PKG system (Fig. 4D).

**NO-induced S-nitrosylation modulates the $Ca^{2+}$ current**

We also investigated whether chemical modification of sulfhydryl groups on $Ca^{2+}$ channels participates in the current inhibition induced by NO donors. For this, the effect of 100 μM SNP was tested in the presence of N-ethylmaleimide (NEM), which is known to covalently modify protein sulfhydryl groups to prevent S-nitrosylation of proteins (Bolotina et al. 1994). NEM (1 mM) was applied intracellularly by the recording pipette. In the presence of intracellular NEM, the effect of 100 μM SNP on the $I_{\text{Ca}}$ was significantly diminished (28 ± 4.5%; $n = 6$, $P = 0.03$ compared with the 53% inhibition by SNP alone) (Fig. 5). In this condition, the effect of the SNP was slightly reversible with respect to the control (38 ± 17%). We also tested the action of 1 mM NEM applied extracellularly by microperfusion. Importantly, in this case NEM by itself produced a significant 90% reduction of the $I_{\text{Ca}}$ and thus no further pharmacological interaction was studied in this condition ($n = 4$; data not shown).

**DISCUSSION**

We found that nitric oxide inhibits $I_{\text{Ca}}$ in hair cells in a voltage-independent manner by the activation of a cGMP-signaling pathway and also by the participation of a direct modification of the $Ca^{2+}$ channel. This is supported because the inhibition of the $I_{\text{Ca}}$ by NO donors was mimicked by a membrane-permeant cGMP analog (8-Br-cGMP) and it was
significantly prevented by an inhibitor of the sGC (ODQ), by an inhibitor of the PKG (KT-5823), and by the presence of an inhibitor of S-nitrosylation (NEM). These results indicate that the actions of NO on the $I_{\text{Ca}}$ imply both an indirect mechanism through a NO–cGMP–PKG pathway and a direct mechanism mediated by S-nitrosylation.

A potential problem with the use of SNP is that in solution it releases cyanide ions that may influence the $I_{\text{Ca}}$ (Biscoe and Duchen 1989). However, the use of NO donors (SIN-1 and NOR-4) that do not produce cyanide indicates that the inhibition of $I_{\text{Ca}}$ is caused by a product common to the three donors: nitric oxide. Additionally, the inhibitory effect of the SNP was blocked by the NO scavenger CPTIO, lending further support to the idea that the effects of the SNP are produced through the NO–cGMP–PKG pathway and a direct mechanism mediated by S-nitrosylation.

The lack of complete reversibility of the $I_{\text{Ca}}$ inhibition by NO donors may be caused by a combination of factors, including the slow deactivation of NO–sGC (Margulis and Sitaramayya 2000) and the S-nitrosylation of the Ca$^{2+}$ channel process, which stabilizes NO in a biologically active form and that may give rise to disulfide-bond formation (Stamler et al. 1992).

The vestibular endorgans one of the targets proposed for NO is the low-threshold K$^{+}$ current ($I_{\text{K,L}}$) expressed in type I hair cells (Rennie and Correia 1994; Rüschi and Eaton 1995a,b). Behrend et al. (1997) found that the cGMP and its membrane-permeant 8-Br-cGMP analog inhibit $I_{\text{K,L}}$. Later it was reported that NO donors inhibit $I_{\text{K,L}}$ through the sGC–cGMP pathway (Chen and Eaton 2000). From these results, inhibition of $I_{\text{K,L}}$ by NO could substantially affect the excitability of the type I hair cells, shifting the membrane potential toward positive voltages and increasing the cell membrane resistance, thus increasing the cell gain and enhancing the transmitter release. However, our results indicate that the action of NO on the posttransductional processing of vestibular information may be more complex. The inhibition of the
voltage-activated Ca\(^{2+}\) channels would decrease the transmitter release in such a way that NO would function as a part of a synaptic-strength modulatory mechanism. This mechanism would act both pre- and postsynaptically. Inhibition of the voltage-activated Ca\(^{2+}\) channels would also decrease the Ca\(^{2+}\)-activated K\(^+\) current, which is expressed both in hair cells and afferent neurons (Hudspeth and Lewis 1988; Limón et al. 2005). Consequently, the overall excitatory or inhibitory effect of NO production on the vestibular activity would depend on the relative functional weight of each of these processes.

Postsynaptically, NO production would depend on NO released from the nerve endings after Ca\(^{2+}\) enters through N-methyl-d-aspartate receptors and voltage-gated Ca\(^{2+}\) channels, and activates NOS I/III (Moncada et al. 1991). Accordingly, NOS I and III were reported in the afferent neurons (Hess et al. 1998a,b). In addition to this, the source of NO might also be presynaptic. Although Hess et al. (1998a,b) found NOS I and III in cochlear but not vestibular hair cells of guinea pig and mice, these enzymes were reported in frog saccular hair cells (Heinrich et al. 2003) and NO production was described in guinea pig vestibular hair cells (Takumida and Anniko 2000). Importantly, the intracellular Ca\(^{2+}\) concentration needed to activate NOS might inhibit SGC (Parkerison et al. 1999), meaning that NO acts on neighboring hair cells but not on the hair cell that generated it (Lysakowski and Singer 2000).

The inhibitory effects of NO on I\(_{K,L}\) and on Ca\(^{2+}\) channels, although apparently contradictory, may reflect the existence of multiple NO-sensitive mechanisms that would control the hair cell response. The inhibition of the Ca\(^{2+}\) current using NO donors and 8-Br-cGMP occurs at the micromolar range, whereas that of the I\(_{K,L}\) current occurs at the millimolar range (Behrend et al. 1997; Chen and Eatock 2000), indicating that the Ca\(^{2+}\) current is much more sensitive to NO than I\(_{K,L}\). We hypothesize that in the vestibular endorgans the action of NO can be dual. At low levels it may have a neuroprotective role constituting an effective mechanism controlling neurotransmitter release from hair cells and at high levels NO may contribute to excitotoxic processes by boosting type I hair cell response (because of I\(_{K,L}\) inhibition). In the vestibular system, nonphysiological stimuli such as the exposure to lipopolysaccharides (Takumida and Anniko 1998), cisplatin (Watanabe et al. 2000), and gentamicin (Takumida and Anniko 1998), meaning that NO acts on neighboring hair cells but not on the hair cell that generated it (Lysakowski and Singer 2000).

Finally, postsynaptic effects of NO should also be considered. NO can inhibit or enhance the Na\(^+\) current (Kawai and Miyachi 2001; Li et al. 1998) and also activate the effect of the large-conductance Ca\(^{2+}\)-activated (BK) channels (Klyachko et al. 2001). Studies in mammals showed that the calyx terminals and vestibular-afferent neurons express a TTX-sensitive Na\(^+\) current (Chabbert et al. 1997; Rennie and Streeter 2006) and that the BK current is also expressed in vestibular-afferent neurons (Limón et al. 2005). Therefore the potential effects of NO on the activity of ionic channels in afferent neurons should also be investigated to attain a complete picture of the participation of NO in the sensory coding of vestibular information. The final result of NO production in the vestibule would be complex depending on the NO levels that are reached and the functional role of pre- and postsynaptic ionic channels that are modulated by NO.

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