Nitric Oxide Inhibits L-Type Ca\(^{2+}\) Current in Glomus Cells of the Rabbit Carotid Body Via a cGMP-Independent Mechanism

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Summers, Beth A., Jeffrey L. Overholt, and Nanduri R. Prabhakar. Nitric oxide inhibits L-type Ca\(^{2+}\) current in glomus cells of the rabbit carotid body via a cGMP-independent mechanism. J. Neurophysiol. 81: 1449–1457, 1999. Previous studies have shown that nitric oxide (NO) inhibits carotid body sensory activity. To begin to understand the cellular mechanisms associated with the actions of NO in the carotid body, we monitored the effects of NO donors on the macroscopic Ca\(^{2+}\) current in glomus cells isolated from rabbit carotid bodies. Experiments were performed on freshly dissociated glomus cells from adult rabbit carotid bodies using the whole cell configuration of the patch-clamp technique. The NO donors sodium nitroprusside (SNP; 600 \(\mu M, n = 7\)) and spermine nitric oxide (SNO; 100 \(\mu M, n = 7\)) inhibited the Ca\(^{2+}\) current in glomus cells in a voltage-independent manner. These effects of NO donors were rapid in onset and peaked within 1 or 2 min. In contrast, the outward K\(^{+}\) current was unaffected by SNP (600 \(\mu M, n = 6\)), indicating that the inhibition by SNP was not a nonspecific membrane effect. 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO; 500 \(\mu M\), an NO scavenger, prevented inhibition of the Ca\(^{2+}\) current by SNP (7), whereas neither superoxide dismutase (SOD; 2,000 U/ml, n = 4), a superoxide scavenger, nor sodium hydrosulfite (SHS; 1 mM, n = 7), a reducing agent, prevented inhibition of the Ca\(^{2+}\) current by SNP. However, SNP inhibition of the Ca\(^{2+}\) current was reversible in the presence of either SOD or SHS. These results suggest that NO itself inhibits Ca\(^{2+}\) current in a reversible manner and that subsequent formation of peroxynitrites results in irreversible inhibition. SNP inhibition of the Ca\(^{2+}\) current was not affected by 30 \(\mu M\) LY 83,583 (7) nor was it mimicked by 600 \(\mu M\) 8-bromoguanosine 3’,5’-cyclic monophosphate (8-BrcGMP; n = 6), suggesting that the effects of NO on the Ca\(^{2+}\) current are mediated, in part, via a cGMP-independent mechanism. N-ethylmaleimide (NEM; 2.5 mM, n = 6) prevented the inhibition of the Ca\(^{2+}\) current by SNP, indicating that SNP is acting via a modification of sulfhydryl groups on Ca\(^{2+}\) channel proteins. Norepinephrine (NE; 10 \(\mu M\)) further inhibited the Ca\(^{2+}\) current in the presence of NEM (n = 7), implying that NEM did not nonspecifically eliminate Ca\(^{2+}\) current modulation. Nisoldipine, an L-type Ca\(^{2+}\) channel blocker (2 \(\mu M, n = 6\)), prevented the inhibition of Ca\(^{2+}\) current by SNP, whereas \(\omega\)-conotoxin GVIA, an N-type Ca\(^{2+}\) channel blocker (1 \(\mu M, n = 9\)), did not prevent the inhibition of Ca\(^{2+}\) current by SNP. These results demonstrate that NO inhibits L-type Ca\(^{2+}\) channels in adult rabbit glomus cells, in part, due to a modification of calcium channel proteins. The inhibition might provide one plausible mechanism for efferent inhibition of carotid body activity by NO.

INTRODUCTION

The carotid bodies are sensory organs that detect changes in arterial oxygen. Hypoxia augments the sensory discharge of the carotid bodies, and the resulting reflexes are crucial for maintaining homeostasis during hypoxemia (see Fitzgerald and Lahiri 1986 for references). The chemoreceptor tissue is composed of neurotransmitter-enriched glomus (type I) cells, which are of neural crest origin, and sustentacular (type II) cells that are glial-like. Currently it is believed that glomus cells, which lie in synaptic apposition with sensory nerve endings, are the initial sites of sensory transduction. Several hypotheses have been proposed for the transduction of the hypoxic stimulus at the carotid bodies (Gonzalez et al. 1992). Whatever the mechanism of transduction, much evidence indicates that neurotransmitters play important roles in sensory transmission at the carotid body (Fidone and Gonzalez 1986; Gonzalez et al. 1992; Prabhakar 1994). Thus it is of considerable importance to understand the cellular mechanisms associated with the actions of neurotransmitters at the carotid body.

Nitric oxide (NO) is a gas molecule with free radical properties that functions as a transmitter molecule in both the peripheral and central nervous systems. It is synthesized during the catalytic conversion of arginine to citrulline by the enzyme nitric oxide synthase (NOS) (Snyder 1992). NO is an unusual neurotransmitter in that it diffuses toward its target cells instead of being stored and released from synaptic vesicles. Recent immunocytochemical studies have shown that NOS is present in both nerve fibers (Prabhakar et al. 1993; Wang et al. 1993) and blood vessels within the carotid body (Grimes et al. 1995; Wang et al. 1993). Inhibitors of NOS augment (Chugh et al. 1994; Prabhakar et al. 1993; Wang et al. 1995), whereas NO donors inhibit, the sensory discharge of the carotid bodies (Wang et al. 1994). Recently, Kline et al. (1998) examined ventilatory responses to hypoxia in neuronal NOS knockout mice. These authors found that respiratory responses to hypoxia are augmented selectively in nNOS knockout mice. These authors found that respiratory responses to hypoxia are augmented selectively in nNOS knockout mice, in part due to exaggerated peripheral chemoreceptor sensitivity, suggesting that NO generated by neuronal NOS is an important physiological regulator of carotid body activity. Furthermore, our previous studies have shown that hypoxia inhibits NOS activity in the carotid body, indicating that NO generation is modulated by oxygen (Prabhakar et al. 1993). Taken together, these studies suggest that NO is an important modulator of carotid body activity and that NO production can be regulated by hypoxia. However, very little is known about the cellular mechanisms associated with the actions of NO in the carotid body.

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There are a number of pathways through which NO can exert its effects. In many systems, NO acts through the activation of heme containing guanylate cyclase and subsequent elevation of cellular levels of cyclic GMP (cGMP) (Snyder 1992). NO signaling also can encompass the actions of other naturally occurring NO derivatives, such as S-nitrosothiols (RSNOs), the actions of which depend on the redox environment of the cell (Campbell et al. 1996; Stamler et al. 1992). Cellular targets of cGMP and/or S-nitrosothiols include channel proteins that regulate ionic conductances. In addition, NO has been shown to directly affect ion channel activity by nitrosylation of sulfhydryl groups on the channel protein (Bolognini et al. 1994; Campbell et al. 1996; Hu et al. 1997). Several studies have shown that NO affects Ca\(^{2+}\) currents in a variety of cells. Regardless of the pathway, reported effects of NO on Ca\(^{2+}\) current are complex and include both inhibition and augmentation. For example, in vascular smooth muscle cells (A7r5 cells), Ca\(^{2+}\) currents are inhibited by NO (Blatter and Wier 1994). On the other hand, NO donors augment Ca\(^{2+}\) currents in neurons of the superior cervical ganglion (Chen and Schofield 1995).

Ca\(^{2+}\) play an important role in carotid body chemoreception. As in other systems, Ca\(^{2+}\) channels are involved in the release of neurotransmitters from glomus cells (Gonzalez et al. 1992). This Ca\(^{2+}\)-dependent neurotransmitter release from glomus cells is believed to be an obligatory step in the chemotransduction process (Biscoe and Duchen 1990; Fidone and Gonzalez 1986; Prabhakar 1992; Shirahata and Fitzgerald 1991). Previous studies have shown that both rat and rabbit glomus cells exhibit high-voltage-activated (HVA), but not low voltage-activated, (LVA) Ca\(^{2+}\) current (e Silva and Lewis 1995; Urena et al. 1989). The HVA Ca\(^{2+}\) current has been shown to be sensitive to dihydropyridines in both rabbit (Obeso et al. 1992) and rat (e Silva and Lewis 1995; Fieber and McCleskey 1993; Peers et al. 1996), suggesting that they express L-type channels. In addition, we recently have reported that the Ca\(^{2+}\) current in rabbit glomus cells is conducted by L, N, P/Q and resistant channel types (Overholt and Prabhakar 1997).

NO, once released from the nerve endings, affects carotid body sensory activity by changing carotid body blood flow via its action on vascular smooth muscle cells and/or by direct action on the glomus cell acting as a retrograde messenger as it does elsewhere in the nervous system (Snyder 1992). Because several lines of evidence indicate that glomus cells are important for sensory transduction at the carotid body, we investigated the cellular action(s) of NO in this cell type. Our hypothesis is that NO affects one or more of the HVA Ca\(^{2+}\) channel types in glomus cells. To test this possibility, we monitored the effects of NO donors on the macroscopic Ca\(^{2+}\) current in glomus cells isolated from rabbit carotid bodies. Our results demonstrate that NO inhibits voltage-gated Ca\(^{2+}\) channels, an effect that primarily is confined to L-type Ca\(^{2+}\) channels. Further the effects of NO on Ca\(^{2+}\) current in glomus cells appears to be a direct action on the channel protein and/or an associated channel protein rather than through a cGMP-dependent mechanism.

**METHODS**

**General procedures**

Experiments were performed on glomus cells freshly isolated from the carotid bodies of adult rabbits killed with CO\(_2\). Individual glomus cells were dissociated enzymatically as described previously (Overholt and Prabhakar 1997). Briefly, carotid bodies were incubated at 37°C in a solution that contained trypsin (type II, 2 mg/ml, Sigma) and collagenase (type IV, 2 mg/ml, Sigma). The composition of the incubation medium was (in mM) 140 NaCl, 5 KCl, 10 HEPS, and 5 glucose, pH 7.2. The carotid body tissue was triturated mechanically with a fire-polished glass pasteur pipette every 10 min. After 30 min of incubation, cells were pelleted after centrifugation at 1,800 g for 5 min. Dissociated cells were resuspended in a 50/50 mixture of Dulbecco’s minimum essential medium (DMEM) and HAM F12 supplemented with penicillin-streptomycin (GIBCO-BRL), insulin, transferrin, selenium (ITS, Sigma), and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a CO\(_2\) incubator and were used within 36 h. All experiments were performed at room temperature.

**Isolation of Ca\(^{2+}\) current**

Ca\(^{2+}\) current was monitored using the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). Pipettes coated with silicone elastomer (Sylgard, Dow Corning) were made from borosilicate glass capillary tubing and had resistances of 4–5 MΩ. Currents were recorded using an Axopatch 200A voltage-clamp amplifier, filtered at 5 kHz and sampled at a frequency of 28.6 kHz using an IBM-compatible computer with a Digidata 1200 interface and pCLAMP software (Axon Instruments). Currents were not leak subtracted. Current-voltage (I-V) relations were elicited from a holding potential of −80 mV using 25-ms steps (5 s between steps) to test potentials over a range of −50 to +70 mV in 10-mV increments. Current at each potential was measured as the average over a 2.5-ms span at the end of the 25-ms step.

Ca\(^{2+}\) current was isolated by using K\(^{+}\)- and Na\(^{+}\)-free intra- and extracellular solutions. The intracellular solution had the following composition (in mM): 115 CsCl, 20 TEA-Cl, 5 MgATP, 0.1 TrisGTP, 5 EGTA, 10 phosphocreatine, and 5 HEPS, and the pH was adjusted to 7.2 with CsOH. The extracellular solution contained (in mM) 140 NaMGCI, 5.4 CsCl, 10 BaCl\(_2\), 10 HEPS, and 11 glucose, and the pH was adjusted to 7.4 with CsOH. The extracellular solution was changed using a fast-flow apparatus consisting of a linear array of borosilicate glass tubes (Overholt and Prabhakar 1997). In these experiments, Ba\(^{2+}\) was the charge carrier. For simplicity, Ba\(^{2+}\) current conducted by Ca\(^{2+}\) channels will be referred to as Ca\(^{2+}\) current. To observe Na\(^{+}\) current to identify a glomus cell, cells first were superfused with an extracellular solution containing Na\(^{+}\) (see solutions for recording K\(^{+}\) current).

Rundown of Ca\(^{2+}\) current and the effects of drugs were monitored using a wash protocol (25 ms step to 0 mV, 10 s between steps). The effects of NO agents were compensated for rundown using a linear wash protocol (25 ms step to 0 mV, 10 s between steps). The intracellular solution had the following composition (in mM): 120 K-glutamate, 20 KCl, 5 MgATP, 0.1 TrisGTP, 5 EGTA,
and 5 HEPES, and pH was adjusted to 7.2 with KOH. The extracellular solution contained (in mM) 140 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 5.5 HEPES, and 11 glucose, and pH was adjusted to 7.4 with NaOH. The effects of drugs were monitored using a wash protocol that consisted of a 100-ms step to +10 mV from a holding potential of −80 mV (with 10 s between steps). These currents did not need to be corrected for rundown. I-V relations were elicited from a holding potential of −80 mV using 75-ms steps (5 s between steps) to test potentials over a range of −50 to +50 mV in 10-mV increments. Current at each potential was measured as the average over a 9-ms span at the end of the 100-ms step.

**Drugs**

Stock solutions of sodium nitroprusside (SNP), spermine-NO (Research Biochemicals International, RBI), 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium (carboxy-PTIO, RBI), superoxide dismutase (SOD, Alexis), and 8-bromoguanosine 3’:5’-cyclic monophosphate sodium salt (8-bromo-cGMP, Sigma) were prepared fresh in extracellular solutions just before each experiment. All NO donor solutions were protected from direct light. LY 83,583 (RBI) and N-ethylmaleimide (NEM, Sigma) were prepared as stock solutions in ethanol. The final concentration of ethanol was 0.1%. In control experiments (n = 4), the ethanol vehicle alone (i.e., without LY or NEM) did not affect the Ca²⁺ current. Nisoldipine (Miles Laboratories) was prepared as a stock solution in polyethylene glycol (MW = 400, Sigma). α-Conotoxin GVIA and norepinephrine were prepared in an aqueous and a 50 mM ascorbic acid stock solution, respectively (RBI). Sodium hydrosulfite (SHS, i.e., sodium dithionite, Sigma) was added to the extracellular solution to reach a final concentration of 1 mM. This solution then was bubbled under an air-tight flask with nitrogen gas for 30 min. The pH was readjusted to 7.4 with NaOH and the pO₂ was measured with a blood gas analyzer (Laboratory Instruments). The pO₂ was monitored routinely and found to be between 35–40 mmHg.

**Data analysis**

All values are presented as means ± SE. Statistical significance was determined by a paired t-test or a one-way ANOVA where appropriate. P values < 0.05 were considered significant.

**RESULTS**

**Identification of glomus cells**

Freshly dissociated cells from carotid bodies contain several cell types, including glomus and type II cells. Previous studies have shown that rabbit glomus cells are excitable cells and they exhibit Ca²⁺, K⁺, and Na⁺ currents. Whereas, type II cells are nonexcitable and exhibit only a small outward K⁺ current but not Na⁺ or Ca²⁺ currents (Urena et al. 1989). At the beginning of each experiment, cells were exposed to an extracellular solution containing Na⁺ and K⁺. Once the presence of Na⁺ current was confirmed, the extracellular solution was switched to a Na⁺/K⁺-free solution to isolate Ca²⁺ current (see METHODS for solutions). Those cells that exhibited Na⁺ and Ca²⁺ currents were considered to be glomus cells.

**Sodium nitroprusside inhibits Ca²⁺ currents in glomus cells**

NO in the carotid bodies is produced primarily in the nerve fibers that innervate the glomus tissue (Chugh et al. 1994; Prabhakar et al. 1993; Wang et al. 1994). However, primary cell cultures from the carotid body do not contain nerve fibers. Therefore it is necessary to administer NO exogenously to study its effect on Ca²⁺ currents in isolated glomus cells. Nitrosyl compounds such as SNP release NO in physiological solutions and are potential tools to study the biological actions of NO (Lipton et al. 1993). An example illustrating the effects of SNP (600 μM, 5 min application) on Ca²⁺ current recorded from a glomus cell is shown in Fig. 1. Examples of whole cell Ca²⁺ current traces before and during exposure to SNP are shown in Fig. 1A. It is obvious from these traces that SNP decreased the amplitude of the Ca²⁺ current. Figure 1A, inset, depicts the same current traces, but the current in the presence of SNP is scaled up to match the current at the end of the step during control to show that SNP had no effect on the rate of activation. The time course for changes in Ca²⁺ current elicited at 0 mV as the extracellular solution is changed to and from one containing 600 μM SNP (same cell as in A). C: average (n = 7), normalized I-V relations recorded before and after a 5-min exposure to 600 μM SNP in the extracellular solution. Currents are corrected for rundown and normalized to the maximum (usually 0 mV) inward control current recorded in individual cells. D: percentage inhibition of control current at 0 mV by 0.1, 0.3, 0.6, and 1.0 mM SNP (n = 7). Percentage inhibition by SNP of the control current was calculated with the following formula: percentage inhibition = (1 − Idrug/Icontrol)100. *Significance with P < 0.05 from control.
SNP does not affect K⁺ currents in glomus cells

To test whether the effect of SNP is a nonspecific membrane effect on all ionic currents in glomus cells, we tested its effects on the outward K⁺ current. We chose this current because it is the most widely studied current in glomus cells and is believed to be sensitive to hypoxia (i.e., Lopez Barneo et al. 1988). An example illustrating the lack of an effect of SNP on the K⁺ current is shown in Fig. 2. Figure 2A shows the current traces elicited by a step to +10 mV before and during exposure to 600 μM SNP. Figure 2B shows the time course for changes in the K⁺ current at +10 mV, and Fig. 2C shows the average (n = 6), normalized I-V relations in the presence and absence of SNP. In contrast to the Ca²⁺ current, the amplitude of the outward K⁺ current was unaffected by SNP in any of the six cells tested. These results demonstrate that SNP does not affect the outward K⁺ current in glomus cells and suggest that the effect of SNP is not a nonspecific membrane effect.

Inhibition of Ca²⁺ current by SNP is not due to cyanide

In addition to NO, SNP also can generate cyanide anions. (CN) (Wink et al. 1996). Biscoe and Duchen (1989) reported that CN⁻ ions inhibit Ca²⁺ current in glomus cells. To establish whether or not the effects of SNP are due to CN⁻ ions, we examined the effect of spermine-NO (SNO; 100 μM), an NO donor that does not generate CN⁻ ions in solution, on the Ca²⁺ current. As shown in the current traces in Fig. 3A, like SNP, SNO decreased the amplitude of the Ca²⁺ current without altering the rate of activation (see inset). Figure 3B shows that the time course of the response to SNO also resembled that elicited by SNP. Furthermore, as with SNP, Ca²⁺ current did not return to control levels within 5 min after washing out SNO. The I-V relation in Fig. 3C shows that, like SNP, SNO inhibited the Ca²⁺ current equally over the range of potentials tested, suggesting the effects are voltage independent. The effects of SNO were seen consistently in all seven cells tested. On average, the Ca²⁺ current was inhibited 21 ± 5% at 0 mV by 100 μM SNO (P < 0.01). Routine use of SNO is difficult because of its short half-life (i.e., 40 min). Therefore SNO was used as the NO donor for the remaining experiments.

To further establish that the effects of SNP are due to generation of NO, we monitored its effects on Ca²⁺ current in the presence of carboxy-PTIO (CPTIO, 500 μM), a potent scavenger of NO (n = 7). It is evident from the current trace shown in Fig. 4A as well as from the time course shown in Fig. 4B that SNP did not inhibit the Ca²⁺ current in the presence of CPTIO. SNP did not have a significant effect on Ca²⁺ current in the presence of CPTIO in any of the seven cells tested. Figure 4B also shows that CPTIO by itself had no effect on Ca²⁺ current. On average, in the presence of CPTIO, SNP inhibited Ca²⁺ current only by 7 ± 5%, which was not significant compared with baseline values (P > 0.05, paired t-test, n = 7; Fig. 4C).

Reversibility of SNP-induced inhibition of the Ca²⁺ current in the presence of SOD and SHS

In the experiments described in the preceding text, calcium currents did not return to controls within 5 min after washing out NO donors (either SNP or SNO). NO, however, is known to generate peroxynitrite in the presence of oxygen by binding
to superoxide ions (Stamler 1994). The following experiments were conducted to test whether peroxynitrites contribute to the lack of reversibility of the response. We examined the effect of SNP on Ca$^{2+}$ current in the presence of superoxide dismutase (SOD 2000 U/ml), a scavenger of superoxide ions ($n = 4$). We reasoned that in the presence of SOD, peroxynitrite formation should be minimal and consequently the effects of SNP on Ca$^{2+}$ currents should be reversible. As shown in the currents in Fig. 5A and the time course in Fig. 5B, SNP inhibited the Ca$^{2+}$ current in the presence of SOD and Ca$^{2+}$ currents returned to the control values within 5 min after washout of SNP in three of the four cells tested. Moreover, the response to SNP in the presence of SOD was qualitatively and quantitatively similar to that in the absence of SOD (compare with Fig. 1, A and B). The average results shown in Fig. 5E show that in the presence of SOD, SNP inhibited the Ca$^{2+}$ current by 25 ± 6%, which is comparable with that seen without SOD (30 ± 6%; $P > 0.05$; unpaired $t$-test).

Peroxynitrite formation is minimal in the presence of reducing agents (Gaston et al. 1993). Therefore, we examined the effect of SNP on the Ca$^{2+}$ current in the presence of 1 mM SHS (i.e., sodium dithionite, PO$_2$ 35–40 mmHg), a reducing agent. As shown in the current traces in Fig. 5C and the time course in Fig. 5D, SNP inhibited the current in the presence of SHS and the effects of SNP were reversible in all seven cells tested. The average results presented in Fig. 5E show that in the presence of SHS, SNP inhibited the Ca$^{2+}$ current by 25 ± 4%, which is comparable with that seen without SHS ($n = 7$, 30 ± 6%, $P > 0.05$, unpaired $t$-test). These observations indicate that peroxynitrites contribute to the lack of the reversibility of Ca$^{2+}$ current inhibition by SNP.

Inhibition of the Ca$^{2+}$ current by NO: evidence for nitrosylation

To test whether SNP inhibition of the Ca$^{2+}$ current is associated with activation of guanylate cyclase, we monitored the effects of SNP on the Ca$^{2+}$ currents in the presence of LY 83,583 (LY), a guanylate cyclase inhibitor. As evidenced by the current traces in Fig. 6A and the time course in Fig. 6B, LY by itself had no effect on the Ca$^{2+}$ current. More importantly, these figures show that LY did not significantly affect the magnitude of the inhibition or alter the time course of the response to SNP. On average, SNP reduced Ca$^{2+}$ current by 20 ± 6% ($n = 7$) and 30 ± 6% ($n = 7$) in the presence and absence of LY, respectively. Although the magnitude of the response tended to be less in the presence of LY, the difference was not statistically significant ($P > 0.05$; unpaired $t$-test).

We reasoned that if the effects of SNP are mediated by cGMP, then 8-Br-cGMP should mimic the effects of SNP on...
the Ca$^{2+}$ current. This possibility was tested in six additional cells. Applications of 8-Br-cGMP (600 μM) for as long as 10 min had no effect on the Ca$^{2+}$ current (data not shown). An example of the raw whole cell Ca$^{2+}$ current traces and the time course of the effects of 8-Br-cGMP on glomus cell Ca$^{2+}$ current are shown in Fig. 6, C and D, respectively. It can be seen that 8-Br-cGMP alone had no effect on Ca$^{2+}$ current even though subsequent application of SNP (600 μM) in the presence of 8-Br-cGMP caused a prompt inhibition of the Ca$^{2+}$ current. Further, the magnitude of SNP-induced inhibition was the same with and without 8-Br-cGMP (29 ± 3% and 30 ± 6%, respectively; P > 0.05, unpaired t-test). Under the present experimental conditions, these results further support the idea that SNP inhibits Ca$^{2+}$ current via a cGMP-independent mechanism(s).

To test whether the NO-induced inhibition of the Ca$^{2+}$ current is due to a modification of sulphydryl groups on Ca$^{2+}$ channels, we examined the effect of SNP in the presence of NEM, which is known to covalently modify sulfhydryl groups of channel proteins and that NO modulates Ca$^{2+}$ channel activity, in part, via a covalent modification of sulphydryl group(s).

SNP-induced inhibition of the Ca$^{2+}$ current is mediated by effects on L-type calcium channels

NO has been shown to primarily affect L-type Ca$^{2+}$ channels in other cells (Campbell et al. 1996; Hu et al. 1997). To test whether the effects of NO are selectively coupled to L-type Ca$^{2+}$ channels in glomus cells, we monitored the effects of SNP on Ca$^{2+}$ current in the presence of 2 μM nisoldipine, a specific L-type Ca$^{2+}$ channel blocker (n = 6). Parallel experiments with 1 μM ω-conotoxin GVIA (CONO), a selective blocker of N-type Ca$^{2+}$ channels, served as controls (n = 9). Figure 8A shows current traces elicited by a step to 0 mV before and during application of either nisoldipine alone or nisoldipine and SNP in the extracellular solution. As expected, nisoldipine by itself blocked a portion of the Ca$^{2+}$ current (37 ± 4%). However, in the presence of nisoldipine, SNP had
latter effects were rapid, occurring within seconds after the
application of NO donors (i.e., Fig. 1B). This pattern of re-
ponse to NO donors is consistent with those reported else-
where in the nervous system (Snyder 1992). The present re-
results, however, are at variance with those reported by others
who found no effect of NO donors on Ca$^{2+}$ currents in rat
glomus cells (Hatton and Peers 1996). One possibility for this
discrepancy is that previous studies employed SNAP as the NO
donor, whereas we used SNP or SNO in the present study.

SNP also may generate CN$^-$ ions in solution (Wink et al. 1996).
Given that CN$^-$ inhibits Ca$^{2+}$ channel activity in glo-
mus cells (Biscoe and Duchen 1989), it could be argued that
the SNP-induced inhibition of the Ca$^{2+}$ current is due to CN$^-$
and not due to NO. Such a possibility, however, seems unlikely
because SNO, a NO donor that does not produce CN$^-$, also
inhibited Ca$^{2+}$ current. In a different series of experiments, we
monitored NO production from SNP by microvoltammetric
 technique using a NO microelectrode (WPI). Using both dif-
f erential pulse voltammetry (DPV) and amperometry, we
found that NO indeed is generated from SNP (N. R. Prabhakar
and M. Gratzl, unpublished observations). These observations,
taken together with the observations that SNP no longer inhib-
it the Ca$^{2+}$ current, the presence of an NO scavenger
(carboxy PTIO), support the idea that the effects of SNP are
indeed due to release of NO. We also do not believe that the
effects of NO donors are nonselective as reported for other
pharmacological substances (e.g., cytochrome P-450 inhibi-
tors) (Hatton and Peers 1996), because conotoxin-sensitive
Ca$^{2+}$ channels (i.e., N type) as well as the outward K$^+$
current were unaffected by SNP. These observations thus demon-
strate that NO donors inhibit Ca$^{2+}$ current in glomus cells from
rabbit carotid bodies and that this effect is due to NO itself not
from byproducts such as CN$^-$. 

A striking finding of the present study is that the effects of
NO donors (both SNP and SNO) on Ca$^{2+}$ current are not
reversible even after 5 min of terminating the challenges.
These observations give an impression that the effects of NO
are irreversible. NO generates peroxynitrates in the presence
of superoxide ions (Stamler 1994) that could potentially affect
cell membranes. We believe that the lack of reversibility of the
response to NO is due to peroxynitrite formation for the fol-
lowing reasons. SNP responses were reversible in the presence
of SOD, a potent scavenger of superoxide ions and SHS, a
strong reducing agent. Both these substances prevent peroxyni-
trite formation. SOD is a cytosolic enzyme, and it is likely that
the effective levels of SOD are expected to be low in our cells
due to dialysis of the cell under the whole cell configuration of
the patch-clamp technique. Therefore under our experimental
conditions, low levels of endogenous SOD would not be able
to effectively scavenge superoxide anions, thereby preventing
consequent generation of peroxynitrite. As expected if this
were the case, exogenous addition of SOD would effectively
reverse SNP-induced inhibition of calcium current. Further,
isolated cells were exposed to atmospheric oxygen (~pO$_2$
= 149 mmHg), wherein generation of superoxide ions is expected
to be high compared with in vivo carotid body where tissue
oxygen levels are expected to be much lower. Thus in intact
carotid bodies, the effects of NO are expected to be reversible
because of high levels of SOD and relatively low tissue oxygen
levels resulting in minimal levels of peroxynitrates. It is im-
portant to note that the magnitude of the inhibition of calcium
currents was unaffected by either SOD or SHS, indicating that
peroxynitrites do not contribute to the suppression of the Ca\(^{2+}\) currents by SNP.

**Mechanisms of calcium channel inhibition by NO in glomus cells**

Considerable evidence indicates that NO exerts many of its effects through the activation of guanylate cyclase and increased levels of cGMP (Snyder 1992). Consistent with such a notion, we (Prabhakar et al. 1993) as well as others (Wang et al. 1989) have reported previously that NO stimulates cGMP levels in the carotid body. On the basis of these observations, we performed several experiments to test whether guanylate cyclase and cGMP are involved in the NO-mediated inhibition of the Ca\(^{2+}\) current in glomus cells. Under the conditions of the whole cell patch configuration, results from these experiments indicate that the effects of NO on calcium currents may, in part, be due to a mechanism independent of cGMP. First, a guanylate cyclase inhibitor, LY 83,583, failed to prevent the SNP-induced inhibition of the Ca\(^{2+}\) current (Fig. 6, A and B). Second, a membrane permeant analogue of cGMP, 8-Br-cGMP, did not mimic the SNP-induced inhibition (Fig. 6, C and D). Third, when SNP was added to glomus cells in the presence of the cGMP analogue, it still caused inhibition of calcium current. Our present observations are in accord with those reported by Hatton and Peers (1996), who also found that both 8-Br-cGMP and PET-cGMP, analogues of cGMP, had no affect on the whole cell Ca\(^{2+}\) current in rat glomus cells. However, the possible involvement of cGMP and the subsequent activation of protein kinase G, cannot be ruled out using the whole cell configuration of the patch-clamp technique because the cell has been dialyzed, allowing escape of small molecules and cytosolic enzymes. Nonetheless, it is possible that NO might directly affect the channel protein or an associated channel protein. Several studies have shown that NO can directly activate or inhibit channel activity through S-nitrosylation. Bolotina et al. (1994) found that NEM, an agent that prevents S-nitrosylation of proteins, prevented activation of Ca\(^{2+}\)-activated \(K^+\) current by NO in vascular smooth muscle cells. Likewise, after NEM treatment, NO donors no longer activated L-type Ca\(^{2+}\) current in ferret ventricular myocytes (Campbell et al. 1996). Hu et al. (1997) reported that specific cysteine modifying agents also could prevent inhibition of the L-type Ca\(^{2+}\) current by NO donors. These observations by other investigators prompted us to test if S-nitrosylation is involved in NO-mediated inhibition of the Ca\(^{2+}\) current in glomus cells. Our results provide evidence that NO is acting through S-nitrosylation in glomus cells. In the presence of NEM, a reagent that prevents S-nitrosylation, SNP did not affect Ca\(^{2+}\) current in glomus cells. We believe that this is not a nonspecific effect of NEM on Ca\(^{2+}\) channel function, because much Ca\(^{2+}\) current remained in the presence of NEM and NO was able to suppress this Ca\(^{2+}\) current remaining. This suggests that the signaling pathway mediating the actions of NE in glomus cells is unaffected by NEM. Taken together, these observations under whole cell conditions provide evidence that inhibition of Ca\(^{2+}\) current by NO in glomus cells involves S-nitrosylation of channel proteins.

**NO inhibition is primarily confined to L-type Ca\(^{2+}\) channels**

It is evident from recent studies that glomus cells express multiple calcium channels (e Silva et al. 1995; Overholt and Prabhakar 1997; Peers et al. 1996). It is well known that neurotransmitters can preferentially affect a specific type of Ca\(^{2+}\) channel within a given cell while sparing others. For instance, we recently have found that NE selectively inhibits a toxin-resistant non-L, N, P/Q type channel in glomus cells of the rabbit carotid body (Overholt and Prabhakar 1998). Therefore, we examined whether the effects of NO are confined preferentially to one type of Ca\(^{2+}\) channel in glomus cells. Our data indicate that the effects of NO are confined to L-type Ca\(^{2+}\) channels in glomus cells. Inhibition of L-type Ca\(^{2+}\) current by NO seen in glomus cells resembles that reported for L-type current in cardiac myocytes (Campbell et al. 1996). Furthermore, NO donors (both SNP and SNO) inhibited the Ca\(^{2+}\) current in every rabbit glomus cell tested, suggesting that the effects of NO are uniform among individual glomus cells.

What might be the physiological importance of NO-induced inhibition of calcium channels in the glomus cells? Several lines of evidence indicate that NO inhibits carotid body activity (Chugh et al. 1994, Kline et al. 1998; Prabhakar et al. 1993; Wang et al. 1995). NOS is expressed in the sinus nerve (Chugh et al. 1994; Prabhakar et al. 1993; Wang et al. 1993) as well as the nerve fibers that originate from the petrosal ganglion (Wang et al. 1993). Stimulation of the sinus nerve inhibits the sensory activity (efferent inhibition) (see Wang et al. 1995 for references). It has been suggested that NO is a mediator of efferent inhibition in the carotid body (Prabhakar et al. 1993; Wang et al. 1995). Previous studies have documented that L-type calcium channels are associated with the release of neurotransmitter(s) in the carotid body (Obeso et al. 1992). Perhaps one of the mechanisms by which NO mediates efferent inhibition is by inhibiting L-type Ca\(^{2+}\) channels in glomus cells and subsequent neurotransmitter release. However, under the conditions of the present experiments, we do not know how much NO is released by NO donors nor do we know the levels of NO released in the carotid body. Therefore further studies are necessary to demonstrate the effects of NO on calcium channels during efferent inhibition of sensory activity.

In summary, the present results demonstrate that NO inhibits L-type voltage-gated Ca\(^{2+}\) channels in adult rabbit glomus cells, in part, via a direct effect on calcium channel protein. It is suggested that inhibition of L-type Ca\(^{2+}\) current in glomus cells would constitute one of the cellular mechanisms associated with actions of NO associated with efferent inhibition of carotid body activity.

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