Nitric Oxide Is an Autocrine Regulator of Na\(^+\) Currents in Axotomized C-Type DRG Neurons

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Renganathan, M., T. R. Cummins, W. N. Hormuzdiar, J. A. Black, and S. G. Waxman. Nitric oxide is an autocrine regulator of Na\(^+\) currents in axotomized C-type DRG neurons. J. Neurophysiol. 83: 2431–2442, 2000. In this study, we examined whether nitric oxide (NO) is upregulated in small dorsal root ganglion (DRG) neurons after axotomy and, if so, whether the upregulation of NO modulates Na\(^+\) currents in these cells. We identified axotomized C-type DRG neurons using a fluorescent label, hydroxysulfamine methanesulfonate and found that sciatic nerve transection upregulates NOS activity in 60% of these neurons. Fast-inactivating tetrodotoxin-sensitive (TTX-S) Na\(^+\) (“fast”) current and slowly inactivating tetrodotoxin-resistant (TTX-R) Na\(^+\) (“slow”) current were present in control noninjured neurons with current densities of 1.08 ± 0.09 nA/pF and 1.03 ± 0.10 nA/pF, respectively (means ± SE). In some control neurons, a persistent TTX-R Na\(^+\) current was observed with current amplitude as much as ~50% of the TTX-S Na\(^+\) current amplitude and 100% of the TTX-R Na\(^+\) current amplitude. Seven to 10 days after axotomy, current density of the fast and slow Na\(^+\) currents was reduced to 0.58 ± 0.05 nA/pF (P < 0.01) and 0.2 ± 0.05 nA/pF (P < 0.001), respectively. Persistent TTX-R Na\(^+\) current was not observed in axotomized neurons. Nitric oxide (NO) produced by the upregulation of NOS can block Na\(^+\) currents. To examine the role of NOS upregulation on the reduction of the three types of Na\(^+\) currents in axotomized neurons, axotomized DRG neurons were incubated with 1 mM \(\text{N}^2\)-nitro-l-arginine methyl ester (l-NAME), a NOS inhibitor. The current density of fast and slow Na\(^+\) channels in these neurons increased to 0.82 ± 0.08 nA/pF (P < 0.01) and 0.34 ± 0.04 nA/pF (P < 0.05), respectively. However, we did not observe any persistent TTX-R current in axotomized neurons incubated with l-NAME. These results demonstrate that endogenous NO/NO-related species block both fast and slow Na\(^+\) current in DRG neurons and suggest that NO functions as an autocrine regulator of Na\(^+\) currents in injured DRG neurons.

INTRODUCTION

Nitric oxide (NO) serves as a cellular mediator for diverse developmental and physiological processes (Bredt and Snyder 1994a; Moncada and Higgs 1993) and is a putative neuromodulator in the brain, spinal cord, and peripheral nervous system (Holscher 1997). Recent evidence suggests that NO may be involved in central (Machelska et al. 1997; Moore et al. 1991; Salter et al. 1996) and peripheral (Haley et al. 1992; Holthusen and Arndt 1995; Ialenti et al. 1992; Larson and Kitto 1995; Lawand et al. 1997; Thomas et al. 1996) pain mechanisms. It is formed by the enzyme NO synthase (NOS), which generates NO from the guanidine group of arginine, giving rise to NO and stoichiometric amounts of citrulline. NO is formed by three different types of NOS derived from three distinct genes referred to, respectively, as neuronal NOS (nNOS; NOS-1), macrophage or inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3) (Moncada et al. 1997). nNOS is localized to discrete populations of neurons in the embryonic and adult nervous system (Bredt and Snyder 1994b). Among the neuronal systems positive for NO/NO-related activity are spinal sensory (dorsal root ganglion, DRG) neurons, the highest number of which are found at the thoracic level, with only a few neurons in the lumbar ganglia in displaying NO-like immunoreactivity in uninjured subjects (Aimi et al. 1991).

After transection of the sciatic nerve in rats, there is a marked increase in the numbers of lumbar DRG neurons containing NOS mRNA, with the higher number of cells (45% of the total) detected after 7 days of injury (Verge et al. 1992). Zhang et al. (1993) reported that in normal adult rats, nNOS is expressed in very few (<4%) small- and medium-diameter DRG neurons. However, a large percentage (~50%) of DRG neurons contain nNOS after peripheral nerve damage (Zhang et al. 1993). These studies did not definitively establish whether the neurons that show upregulation of NOS activity are the neurons the axons of which were transected at the level of sciatic nerve. Because only ~54% of the cells in the L4 and L5 DRGs project an axon into the sciatic nerve (Devor et al. 1985; Yip et al. 1984), it is important to establish whether NOS is upregulated specifically in the axotomized lumbar ganglia neurons.

Earlier studies from our laboratory established that, after injury to their axons, C-type DRG neurons display changes in sodium channel expression that include a downregulation of tetrodotoxin (TTX)-resistant (TTX-R) sodium currents and upregulation of a rapidly repriming TTX-sensitive (TTX-S) Na\(^+\) current (Cummins and Waxman 1997). However, despite the evidence that axotomy upregulates nNOS in DRG neurons, the role of NO in the modulation of Na\(^+\) currents in these cells has not been established. NO, when experimentally applied to tissues via the use of nitric oxide donors, causes reversible conduction block in both normal and demyelinated axons of the central and peripheral nervous system (Redford et al. 1997; Shrager et al. 1998). In baroreceptor neurons, endogenous NO as well as exogenously added NO donors have been shown to inhibit TTX-S and TTX-R Na\(^+\) currents (Li et al. 1998). These studies suggest that endogenous NO/NO-related activity might...
alter DRG neuron electrical excitability by modulating Na\(^+\) currents.

In this study, we focused on small DRG neurons, which are involved in nociceptive signaling, and asked whether NO is upregulated after axotomy and whether upregulation of NOS blocks Na\(^+\) currents in axotomized neurons. DRG neurons the axons of which had been transected were identified with a retrogradely transported fluorescent label, hydroxystilbamine methanesulfonate. Assay of axotomized C-type DRG neurons for NOS activity with nNOS specific antibody revealed up-regulation of NOS in \(\geq 60\%\) of cells. Exposure of axotomized type-C DRG neurons to a NOS inhibitor led to a significantly higher density of fast and slow Na\(^+\) currents, demonstrating that endogenous NO/NO-related species modulate these Na\(^+\) currents in axotomized C-type DRG neurons and suggesting that NO is an autocrine regulator of Na\(^+\) currents in these cells.

**METHODS**

**Animal care**

3-mo old Sprague-Dawley female rats (120 g weight) were used for the study. Animals were fed ad libitum and housed in a pathogen-free area at the Veterans Affairs Medical Center (VAMC), West Haven. Animal care and surgical procedures followed an approved protocol by the Animal Care and Use Committee of Yale University.

**Sciatic nerve axotomy**

Axotomy of the sciatic nerve was performed as previously described (Waxman et al. 1994). Animals were anesthetized with ketamine/xylazine (38/5 mg/kg ip), and the right sciatic nerve was exposed and a tight ligature was placed around the sciatic nerve near the sciatic notch proximal to the pyriform ligament. The nerve was transected immediately distal to the ligature site, and the proximal nerve stump was fit into a silicone cuff to avoid nerve regeneration. The cuff contained 4 \(\mu\)l of hydroxystilbamine methanesulfonate (4% wt/vol in distilled water; Molecular Probes, Eugene, OR) for retrograde labeling of axotomized neurons. The fluorescent label clearly visualized neurons treated in identical manners. Control experiments included incubation without primary antibody; only background levels of fluorescence were detected in the control experiments.

**Electrophysiological recordings**

Coverslips were mounted in a small flow-through chamber positioned on the stage of a Nikon Diaphot microscope (Nikon) and were perfused continuously with the bath-external solution (see following text) with a push-pull syringe pump (WPI, Saratoga, FL). Hydroxystilbamine-methanesulfonate-labeled neurons were identified by fluorescence emission 510 nm. A short arc mercury lamp was used for the excitation light source and it was defined by broadband (450–490 nm) pass filter (Nikon).

Cells were voltage-clamped via the whole cell configuration of the patch clamp with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) using standard techniques (Hamill et al. 1981). Micropipettes were pulled from borosilicate glasses (Boralex) with aforge (Narishige, Tokyo) to obtain electrode resistance ranging from 30–60 MΩ. The pipette solution contained (in mM): 140 CsF, 1 EGTA, 10 NaCl, 2 CaCl\(_2\), and 20 HEPES, pH 7.3. The following bath solution was used (in mM): 140 NaCl, 3 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 0.1 CdCl\(_2\), and 20 HEPES, pH 7.3. CdCl\(_2\) was used to block Ca\(^{2+}\) current. The pipette potential was zeroed before seal formation, and the voltages were not corrected for liquid junction potential. Capacity transients were cancelled and series resistance was compensated (>80%) when needed. The leakage current was digitally subtracted on-line using hyperpolarizing control pulses of one-sixth test pulse amplitude (–P/6 procedure), and the pulses were applied before the test pulse. Access resistance was monitored throughout the recording and the cells were discarded if the resistance was >5 MΩ. Cells also were discarded if the leakage current was >1.0 nA (holding current >1.0 nA at –100 mV). Whole cell currents were filtered at 5 kHz and acquired at 50 kHz in a computer using Clampex 8.01 software (Axon Instruments). Digidata

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and slow TTX-R Na$^+$ currents. Densities for slow TTX-R Na$^+$ currents were calculated from cells that express only TTX-R Na$^+$ currents and from cells expressing both fast TTX-S and slow TTX-R Na$^+$ currents.

NOS inhibitor

N$^3$ nitro-L-arginine methyl ester (L-NAME), a NOS-specific inhibitor (Sigma Chemicals) was used to investigate whether the upregulation of NOS in axotomized DRG neurons attenuates Na$^+$ current. DRG neurons were incubated overnight with 1 mM L-NAME in the culture medium. L-NAME also was included in the bath and pipette solutions during the recording of Na$^+$ currents.

RESULTS

In this study on the effects of peripheral nerve injury on the upregulation of NOS and the associated modulation of Na$^+$ current, we focused on type-C DRG neurons (<30-μm diam), which have been well characterized after axotomy (Cummins and Waxman 1997). We selected DRG neurons from rats 7–14 days post axotomy (DPA), because a higher number of DRG neurons display a marked increase in NOS mRNA after 7 days of injury (Verge et al. 1992), and a dramatic downregulation of TTX-R Na$^+$ currents occurs after 6 DPA (Cummins and Waxman 1997). Neurons cultured from the uninjured left L4 and L5 DRG neurons of each rat served as controls (106 cells were studied from 12 different cultures). Neurons cultured from the injured right L4 and L5 DRG of each rat were used to study the effects of sciatic nerve transection; 126 axotomized cells from 12 different cultures were studied.

NOS activity is upregulated in axotomized C-type DRG neurons

Figure 1A shows the optical image of a representative group of control DRG neurons, and none of these neurons showed NOS activity when assayed by immunocytochemistry (Fig. 1B). Axotomized lumbar ganglion cells the axons of which were transected at midthigh level of sciatic nerve were identified by retrograde fluorescent dye labeling (Fig. 2A). Upregulation of NOS activity in these axotomized neurons was determined by immunocytochemical reaction (Fig. 2B). To determine whether NOS activity is specifically upregulated in axotomized neurons, images from retrograde fluorescent dye labeling were

![Image](https://example.com/image1.png)

**FIG. 1.** Control nonaxotomized neurons do not show nitric oxide synthase (NOS) immunoreactivity. A: Nomarski image of isolated control dorsal root ganglion (DRG) neurons from L4 and L5 ganglia. B: fluorescence photomicrograph of control neurons assayed for NOS immunoreactivity.

1200B interface (Axon Instruments) was used for A-D conversion, and the data were stored on compact disk for analysis. For current density measurements, membrane currents were normalized to membrane capacitance. Membrane capacitance was calculated as the integral of the transient current in response to a brief hyperpolarizing pulse from $-120 \text{ mV}$ (holding potential) to $-130 \text{ mV}$. The average of the membrane capacitance of the DRG neurons used for electrophysiological experiments was $23.86 \pm 0.89 \text{ pF (n = 106)}$ for control and $32.18 \pm 1.54 \text{ pF (n = 126; P < 0.001)}$ for axotomized DRG neurons.

A Boltzmann function, Availability = $1/(1 + \exp[(V - V_h)/k_n])$, where $V_p$ is the prepulse potential, $V_h$ is the midpoint potential, and $k_n$ is the corresponding slope factor for Boltzmann function, was used to fit inactivation-voltage relationship.

Calculation of fast and slow Na$^+$ current density using prepulse inactivation

Prepulse inactivation takes advantage of the differences in the inactivation properties of the fast and slow Na$^+$ currents (Cummins and Waxman 1997; MeLean et al. 1988; Roy and Narahashi 1992). The currents were elicited by 20-ms test pulses to $-10 \text{ mV}$ after 500-ms prepulses to potentials over the range of $-130$ to $-10 \text{ mV}$. The fast TTX-S Na$^+$ currents were obtained by subtracting the current obtained at $-50 \text{ mV}$ prepulse (only slow TTX-R Na$^+$ current) from the current obtained with more hyperpolarizing prepulses (fast TTX-S and slow TTX-R Na$^+$ currents). Densities for fast TTX-S Na$^+$ currents were calculated from cells that express only TTX-S Na$^+$ cur-

![Image](https://example.com/image2.png)

**FIG. 2.** Axotomized neurons predominantly show NOS immunoreactivity. A: fluorescent dye labeling of axotomized neurons. B: fluorescence photomicrograph of axotomized neurons assayed for NOS immunoreactivity. Images from A and B are superimposed in C. Axotomized neurons showing both fluorescent dye labeling and NOS immunoreactivity appear yellow. Axotomized neurons that do not display NOS appear green. Unlabeled neurons with NOS immunoreactivity appear red.
overlaid on top of the images of the NOS activity; the superimposed images are shown in Fig. 2C. The retrogradely labeled neurons that show upregulation of NOS appear yellow in color, labeled neurons that do not show upregulation of NOS appear green in color, and uninjured neurons that show upregulation of NOS activity appear red in color. Upregulation of NOS activity was seen in 60% of the retrogradely labeled axotomized neurons (87 of 145 neurons from 2 animals).

We observed NOS activity in 26% of the unlabeled neurons, which did not show fluorescent signal in cultures derived from axotomized rats (12 of 45 neurons). Only ~60% of L_4 and L_5 DRG neurons project into the sciatic nerve; ~40% contribute to more proximal nerves and are uninjured after sciatic nerve transection. Unlabeled NOS-positive neurons could have been injured but failed to take up the fluorescent dye. Alternatively, NOS might be upregulated in nonaxotomized neurons by a diffusible factor produced in nearby axotomized neurons.

**Na\(^+\) currents in type-C DRG neurons**

Sodium currents were recorded from type-C (<30 \(\mu\)m) L_4 and L_5 DRG neurons with whole cell patch-clamp techniques. Only fluorescent neurons, which we could identify definitively as axotomized neurons, were studied. The Na\(^+\) currents in control neurons, in cultures from nonaxotomized DRG, were similar to those previously described in small DRG neurons (Caffrey et al. 1992; Cummins and Waxman 1997; Cummins et al. 1999; Elliott and Elliott 1993; Rizzo et al. 1994). On the basis of their inactivation properties, Na\(^+\) currents in type-C DRG neurons can be classified into fast-inactivating, slowly inactivating, and noninactivating Na\(^+\) currents (Cummins and Waxman 1997; Cummins et al. 1999), which we refer to as "fast," "slow," and "persistent" Na\(^+\) currents, respectively. The fast-inactivating currents in both control and axotomized DRG neurons were sensitive to nanomolar concentrations of TTX, whereas the slowly inactivating and the persistent Na\(^+\) currents were resistant to TTX (see following text and Fig. 6). The observation that fast-inactivating Na\(^+\) currents are sensitive and the slow-inactivating and persistent Na\(^+\) currents are resistant to nanomolar concentrations of TTX is consistent with earlier studies (Cummins and Waxman 1997; Cummins et al. 1999; Elliott and Elliott 1993; Roy and Narahashi 1992). Prepulse inactivation was used to separate the fast and slow Na\(^+\) currents (Cummins and Waxman 1997; MeLean et al. 1988; Roy and Narahashi 1992). Prepulse inactivation takes advantage of the differences in the inactivation properties of the fast and slow Na\(^+\) currents and is simpler than TTX subtraction. TTX subtraction and prepulse inactivation have been shown to give essentially the same results when used to separate fast and slow Na\(^+\) currents (Cummins and Waxman 1997). It has been demonstrated, using SNS-null mutant mice that the slow Na\(^+\) current in small DRG neurons is encoded by the SNS sodium channel isoform (Akopian et al. 1999). However, SNS-null and wild-type DRG neurons also express a distinct, non-SNS, TTX-R Na\(^+\) current (Cummins et al. 1999). We refer to this distinct TTX-R Na\(^+\) current as the persistent current to differentiate it from the slow TTX-R Na\(^+\) current. Two criteria were used to separate persistent TTX-R Na\(^+\) currents from the fast and slow Na\(^+\) currents. Persistent currents activate at low threshold depolarizing potentials (about ~80 mV) compared with the fast and slow Na\(^+\) currents, which activate at higher depolarizing potentials (about ~40 mV); and at depolarizing potentials between ~80 to ~40 mV the persistent currents are noninactivating (see following text and Fig. 6 and 7).

**Fast and slow Na\(^+\) current density are reduced in axotomized neurons**

To quantitate fast and slow Na\(^+\) currents expressed in each neuron, prepulse inactivation (MeLean et al. 1988; Roy and Narahashi 1992) was used, and the current amplitudes were measured. The fast and slow current amplitudes were 25.40 ± 2.61 and 26.17 ± 2.83 in control neurons and 19.77 ± 3.81 (\(P < 0.04\)) and 6.41 ± 1.39 (\(P < 0.0001\)) in axotomized neurons. To compensate for differences in cell size, currents were normalized to cell capacitance and expressed as current density. The fast TTX-S and slow TTX-R Na\(^+\) current densities in control neurons were 1.08 ± 0.08 and 1.03 ± 0.10 nA/pF, respectively. Axotomy significantly reduced the fast and slow Na\(^+\) current densities to 0.58 ± 0.05 (\(P < 0.01\)) and 0.20 ± 0.05 nA/pF (\(P < 0.001\)), respectively (Fig. 3, A and B). The cell capacitance in axotomized cells was 32.06 ± 1.43 pF, 32% higher than in control cells where it was 23.86 ± 0.89 pF (Fig. 3C, \(P < 0.01\)). An increase in capacitance of axotomized neurons is expected because axotomized neurons sprout neurites more rapidly than control neurons in culture (Lankford et al. 1998). The increase in cell capacitance (32%), which reflects increased cell membrane area in axotomized neurons, may be responsible for part of the observed reduction in fast Na\(^+\) current density (which was reduced by 46% in axotomized cells compared with controls) and slow Na\(^+\) current density (which was reduced by 81% in axotomized cells compared with controls).

**\(\text{L-NAME increases fast and slow Na}^+\text{ current densities in axotomized neurons}\)**

To investigate whether upregulation of NOS in axotomized DRG neurons attenuates Na\(^+\) currents, DRG neurons were incubated with 1 mM \(\text{L-NAME}\), a NOS inhibitor, overnight in the culture medium. Although, acute application of NOS inhibitor to DRG cells via bath and pipette solutions slightly increased fast TTX-S and slow TTX-R Na\(^+\) current density, it was twofold lesser than the increase observed in overnight incubation of cultured DRG cells with \(\text{L-NAME}\) and including \(\text{L-NAME}\) in bath and pipette solutions. Incubation of control DRG neurons with \(\text{L-NAME}\) did not increase the density of fast or slow Na\(^+\) currents (Fig. 3, A and B); in these neurons the fast or slow Na\(^+\) current densities were 1.02 ± 0.08 and 1.25 ± 0.03 (\(P < 0.05\)), respectively. In contrast, incubation of axotomized neurons with \(\text{L-NAME}\) significantly increased both fast and slow Na\(^+\) current density to 1.87 ± 0.08 (\(P < 0.01\)) and 0.89 ± 0.08 (\(P < 0.01\)), respectively (Fig. 3C, A and B). The respective current amplitudes to 25.96 ± 2.86 (\(P < 0.01\)) and 6.40 ± 1.39 (\(P < 0.0001\)) in axotomized neurons. To compensate for differences in cell size, currents were normalized to cell capacitance and expressed as current density. The fast TTX-S and slow TTX-R Na\(^+\) current densities in control neurons were 1.08 ± 0.08 and 1.03 ± 0.10 nA/pF, respectively. Axotomy significantly reduced the fast and slow Na\(^+\) current densities to 0.58 ± 0.05 (\(P < 0.01\)) and 0.20 ± 0.05 nA/pF (\(P < 0.001\)), respectively (Fig. 3, A and B). The cell capacitance in axotomized cells was 32.06 ± 1.43 pF, 32% higher than in control cells where it was 23.86 ± 0.89 pF (Fig. 3C, \(P < 0.01\)). An increase in capacitance of axotomized neurons is expected because axotomized neurons sprout neurites more rapidly than control neurons in culture (Lankford et al. 1998). The increase in cell capacitance (32%), which reflects increased cell membrane area in axotomized neurons, may be responsible for part of the observed reduction in fast Na\(^+\) current density (which was reduced by 46% in axotomized cells compared with controls) and slow Na\(^+\) current density (which was reduced by 81% in axotomized cells compared with controls).
L-NAME increases the number of cells coexpressing fast and slow Na\textsuperscript{+} currents in axotomized neurons

The proportion of cells expressing fast and slow Na\textsuperscript{+} currents in control cultures were 85 and 70%, respectively, with 60% of control cells coexpressing fast and slow Na\textsuperscript{+} currents. Axotomy reduced the number of neurons expressing slow Na\textsuperscript{+} currents to 20% and increased the number of neurons expressing fast Na\textsuperscript{+} currents to ~40%. However, incubation of control neurons with L-NAME did not significantly increase the number of cells coexpressing fast TTX-S and slow TTX-R Na\textsuperscript{+} currents (Table 1). The increase in the proportion of axotomized neurons expressing slow Na\textsuperscript{+} channels after exposure to L-NAME provides additional evidence that upregulation of NOS in axotomized neurons blocks Na\textsuperscript{+} currents.

Voltage dependence of steady-state inactivation of Na\textsuperscript{+} currents is not altered by axotomy

A majority of the control neurons coexpress fast and slow Na\textsuperscript{+} currents (Table 1; Fig. 4A), and the voltage dependence of inactivation of the Na\textsuperscript{+} currents in these neurons showed a bimodal distribution because of the different inactivation properties of the fast and slow Na\textsuperscript{+} currents (Fig. 4B). The midpoint of steady-state inactivation for fast and slow currents in control neurons was about −74.6 ± 7.4 and −33.7 ± 6.5 mV, respectively (Table 2). These values are similar to the values reported in earlier studies (Caffrey et al. 1992; Cummins and Waxman 1997; Elliott and Elliott 1993; Rizzo et al. 1994; Roy and Narahashi 1992; Rush et al. 1998). Axotomy reduced the amplitude of slow Na\textsuperscript{+} currents (note the absence of slow-inactivating current in Fig. 4D). The voltage dependence of inactivation of the Na\textsuperscript{+} currents in most axotomized neurons could be fit with a single Boltzmann distribution indicative of the presence of only one type of Na\textsuperscript{+} current, i.e., fast Na\textsuperscript{+} current (Fig. 4E). However, slow Na\textsuperscript{+} current was observed in 25% of the axotomized neurons (Table 1). Despite the small size of these currents (less than −5 nA in most cells) we were able to measure their inactivation to the nearest 0.5 mV. The midpoint of steady-state inactivation for fast and slow currents in these neurons was −70.0 ± 6.8 and −34.0 ± 5.2 mV, respectively, similar to the values seen in control neurons (Table 2). These values are similar to the values reported in an earlier study for axotomized type-C neurons (Cummins and Waxman 1997).

**TABLE 1. Effect of sciatic nerve transection and L-NAME on the distribution of fast and slow Na\textsuperscript{+} currents in axotomized C-type DRG neurons**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Fast</th>
<th>Fast and slow</th>
<th>Slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control neurons</td>
<td>63</td>
<td>25</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Control neurons + L-NAME</td>
<td>43</td>
<td>20</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Axotomized neurons</td>
<td>71</td>
<td>78</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Axotomized neurons + L-NAME</td>
<td>75</td>
<td>60</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Values are percentages of the total.
Voltage dependence of steady-state inactivation of Na\textsuperscript{+} currents is not altered by L-NAME

After incubation with L-NAME, 40% of the axotomized neurons showed a bimodal distribution for the voltage dependence of inactivation, indicating the presence of both fast and slow Na\textsuperscript{+} currents in these neurons (Table 1 and Fig. 5, A and B). The remaining 60% of the neurons had midpoint potentials similar to those of fast Na\textsuperscript{+} currents (Fig. 5E). The midpoint of steady-state voltage-dependent inactivation for fast and slow Na\textsuperscript{+} currents in axotomized neurons incubated with L-NAME was \(-73.3 \pm 7.6\) and \(-34.4 \pm 4\), respectively (Table 2). The voltage dependence of inactivation for control neurons incubated with L-NAME was \(-72.7 \pm 5.5\) and \(-33.0 \pm 6.1\) mV, respectively, for fast and slow Na\textsuperscript{+} currents (Table 2). The small differences in the midpoints of steady-state voltage-dependent inactivation for fast and slow Na\textsuperscript{+} currents in control, axotomized, L-NAME-incubated axotomized neurons were not statistically significant, indicating that exposure to NOS does not alter steady-state inactivation. These results, therefore suggest that the same populations of fast and slow Na\textsuperscript{+} channels are subject to potential modulation by NOS in control and axotomized DRG neurons.

**Recovery from inactivation**

We previously have shown that axotomy of small DRG neurons is followed by the emergence of a distinct fast TTX-S Na\textsuperscript{+} current that shows a rapid recovery from inactivation (Cummins and Waxman 1997). Therefore we examined the recovery from inactivation at \(-100\) mV of the fast Na\textsuperscript{+} channels in control, axotomized, and L-NAME-treated control and axotomized neurons. Recovery-from-inactivation curves for typical control and axotomized neurons are shown in Fig. 4, C.

**TABLE 2. Voltage dependence of inactivation in control, axotomized, L-NAME-incubated control and axotomized small C-type neurons**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>(V_h) for Fast Na\textsuperscript{+} Current, mV</th>
<th>(V_h) for Slow Na\textsuperscript{+} Current, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63</td>
<td>(-74.6 \pm 7.4)</td>
<td>(-33.7 \pm 6.5)</td>
</tr>
<tr>
<td>Control + L-NAME</td>
<td>43</td>
<td>(-72.7 \pm 5.5)</td>
<td>(-33.0 \pm 6.1)</td>
</tr>
<tr>
<td>Axotomized</td>
<td>71</td>
<td>(-70.0 \pm 6.8)</td>
<td>(-34.0 \pm 5.2)</td>
</tr>
<tr>
<td>Axotomized + L-NAME</td>
<td>75</td>
<td>(-73.3 \pm 7.6)</td>
<td>(-34.4 \pm 4.0)</td>
</tr>
</tbody>
</table>

Data are expressed as means \(\pm\) SD.

FIG. 4. Voltage dependence of inactivation of Na\textsuperscript{+} currents in axotomized DRG neurons. *Left*: families of traces from representative control and axotomized neurons are shown. Currents were elicited from the respective neurons by 40-ms test pulses to \(-10\) mV after 500-ms prepulses to potentials over the range of \(-130\) to \(-10\) mV. *Middle*: corresponding voltage dependence of inactivation curves are shown for each cell. Current values at different potentials are normalized to peak current value observed at \(-130\)-mV holding potential. Voltage dependence of inactivation in control neurons show a double-Boltzmann distribution. Axotomized neurons that express only fast-inactivating currents show a single-Boltzmann distribution. *Right*: recovery from inactivation is shown for each cell. Respective neurons were held at \(-100\) mV, stepped to 0 mV for 20 ms to inactivate the channels and then brought back to \(-100\) mV for increasing intervals to allow the channels to recover from inactivation, and the recovery was determined by a 20-ms test pulse to 0 mV.
and F. Control neurons treated with L-NAME displayed recovery from inactivation that was similar to that of untreated control neurons. The time course of recovery from inactivation in control neurons could be fit with two exponentials. The rapidly recovering component showed complete recovery within 10 ms and the slowly recovering component showed recovery in 100 ms (Fig. 4C). TTX was used to determine that the rapidly recovering component corresponds to slow TTX-R Na\(^+\) current and the slowly recovering component to a fast TTX-S Na\(^+\) current (results not shown). These results are similar to the results reported earlier (Cummins and Waxman 1997; Elliott and Elliott 1993). Eighty percent of the axotomized neurons expressed only fast TTX-S Na\(^+\) current, and in these neurons, >90% of the recovery from inactivation was seen in ∼10 ms (Fig. 4F). Because these neurons had only TTX-S Na\(^+\) current, the results suggest that the recovery from inactivation of TTX-S Na\(^+\) current in axotomized neurons is rapid and faster than in the uninjured neurons, consistent with the results obtained in an earlier report (Cummins and Waxman 1997). Twenty percent of the axotomized neurons expressed both fast TTX-S and slow TTX-R Na\(^+\) currents, and these currents recovered from inactivation in ∼10 ms.

Both fast TTX-S and slow TTX-R Na\(^+\) current were seen in 40% of L-NAME-incubated axotomized neurons, and complete recovery from inactivation also was seen in ∼10 ms in these neurons (Fig. 5C). The other 60% of the axotomized neurons incubated with L-NAME, which expressed only the fast TTX-S Na\(^+\) currents, also showed >85% recovery from inactivation in ∼10 ms (Fig. 5F). The rapid recovery from inactivation of the fast TTX-S currents in axotomized neurons compared with control neurons suggests that fast TTX-S Na\(^+\) current in axotomized neurons may arise from a different type of Na\(^+\) channel that is expressed in these cells. The similar time course for recovery of the Na\(^+\) currents from axotomized neurons incubated with L-NAME suggests that the channels, which underlie these currents, are similar to those in untreated axotomized neurons. L-NAME thus appears to have little effect on recovery from inactivation of the fast TTX-S and slow TTX-R channels.

Persistent TTX-R Na\(^+\) currents are decreased after axotomy

As described by Cummins et al. (1999) in wild-type and SNS-null mutant mice, when control DRG neurons were held
at −130 mV, persistent TTX-R currents (defined as the current remaining at the end of ≥40 ms depolarizing pulses) were seen in 24 of 40 cells, with current amplitudes ranging from about −40 to about −5 nA. The amplitude of the persistent current in control DRG neurons was 10–50% of the amplitude of fast TTX-S Na⁺ currents and 50–100% of the amplitude of slow TTX-R Na⁺ currents (Fig. 6, A and B). In these experiments, the bath solution contained 100 μM Cd²⁺, a concentration high enough to block low-voltage-activated T-type Ca²⁺ currents. Figure 6A shows the presence of fast TTX-S Na⁺ current and slow TTX-R Na⁺ current together with a persistent current that is present at the end of 40-ms test pulses; the persistent currents manifest as tail currents on returning to a holding potential of −130 mV. Even when measured at the end of 100 ms and in the presence of 300 nM TTX, these persistent currents can be seen (Fig. 6B). Activation of fast TTX-S Na⁺ currents and slow TTX-R Na⁺ currents occurs at about −40 mV (Fig. 7, B and C) consistent with the results obtained in earlier studies (Elliott and Elliott 1993; Rush et al. 1998). In contrast, activation of the persistent Na⁺ currents is seen at about −80 mV (Fig. 7D) similar to the TTX-R persistent current observed by Cummins et al. (1999) in wild-type and SNS-null mutant mice. Further, at test potentials between −80 and −40 mV, the persistent currents are noninactivating, similar to the observation by Cummins et al. (1999) in wild-type and SNS-null mutant mice. Therefore the Na⁺ currents observed between −80 and −40 mV are TTX-R persistent currents.

The peak current amplitude of the current traces shown in Fig. 6, A and B, was measured and depicted as current-voltage plot in Fig. 8, A and B (○). Activation of inward Na⁺ current is seen at −80 mV, reaches a plateau or peak at −40 mV (note the hump at −40 mV), with a peak at −15 mV. Axotomy almost completely abolished the persistent current as seen in the traces in Fig. 6, C and D (note the absence of tail currents), and in the loss of amplitude of the persistent current at test potentials between −80 and −40 mV (Fig. 8, A and B, ●). Very few cells (<10% of the cells) showed persistent current after axotomy, and the maximum amplitude of the persistent current seen in these cells was <5 nA (Fig. 6D). These results are similar to the results obtained in an earlier study (Cummins and Waxman 1997). After incubation with L-NAME, the axotomized neurons show an increase in fast and slow Na⁺ current amplitude but did not show any significant change in the amplitude of the persistent current (Fig. 6, E and F). This also can be seen in Fig. 8, A and B (■), where incubation in L-NAME results in an increase in the currents activated positive to −40 mV, but not in the persistent currents activated between −80 and −40 mV. These results suggest that the decrease in the persistent Na⁺ current amplitude in axotomized neurons is not due to the upregulation of NOS (see DISCUSSION).

**DISCUSSION**

The present study reports the first evidence that upregulation of NOS is seen predominantly in axotomized C-type neurons
and supports the hypothesis that NO derived from NOS is a modulator of Na\textsuperscript{+} currents in these cells after axotomy. This conclusion is supported by our findings that the NOS inhibitor L-NAME increased Na\textsuperscript{+} current density of both fast TTX-S and slow TTX-R Na\textsuperscript{+} current in axotomized C-type DRG neurons but not in control nonaxotomized neurons. These results indicate a role for NO in suppressing fast and slow Na\textsuperscript{+} currents in damaged neurons at the ganglionic level after axotomy and suggest that NO may be an autocrine regulator of Na\textsuperscript{+} currents in C-type DRG neurons.

Our observation that NOS activity was increased in the ipsilateral DRG at the level of nerve injury is consistent with
previous observations suggesting a peripheral role for NO in altering the excitability of injured DRG neurons (Choi et al. 1996; Ferreira et al. 1991; Fiallos-Estrada et al. 1993; Steel et al. 1994; Verge et al. 1992). Using fluorescent dye to identify the axotomized neuron and nNOS-specific antibody to identify NOS activity, we demonstrate that ~60% of retrogradely labeled C-type neurons within L4 and L5 DRG show upregulation of NOS activity after axotomy within the sciatic nerve.

Axotomy results in dramatic and complex changes in the sodium currents expressed in C-type DRG neurons (Cummins and Waxman 1997). Our observation that axotomy decreased the density of slow Na\(^+\) current density to 0.2 nA/pF in C-type DRG neurons is consistent with the previous observations that axotomy triggers decreases in slow Na\(^+\) current density (Cummins and Waxman 1997) and in the steady-state levels of alpha-SNS mRNA, which encodes a slow TTX-R current in these cells (Dib-Hajj et al. 1996). The present results are also consistent with earlier studies that showed loss in axotomized neurons of TTX-R-persistent currents (Cummins and Waxman 1997) and of the NaN transcript (Dib-Hajj et al. 1998), which appears to encode the channels that produce this current (Cummins et al. 1999). However, our observation that axotomy reduced fast TTX-S Na\(^+\) current density to 0.6 nA/pF differs from an earlier study (Cummins and Waxman 1997), which did not report a significant reduction in TTX-S Na\(^+\) current density after axotomy. This difference may be attributed to the difference in cell capacitance of axotomized neurons observed between this study and the earlier study (Cummins and Waxman 1997). We examined DRG neurons 16–24 h after dissociation and observed a 30% increase in cell capacitance after axotomy; however, the earlier study, which examined DRG neurons 10–16 h after dissociation, did not find a significant change in cell capacitance after axotomy (Cummins and Waxman 1997). It recently has been shown that sciatic nerve ligation significantly can enhance neurite growth in isolated DRG neurons (Lankford et al. 1998). This enhancement probably accounts for the increased capacitance and decreased TTX-S current density observed in axotomized neurons studied at 16–24 h in vitro (this study) but not at 10–16 h in vitro after axotomy (Cummins and Waxman 1997). In this study, DRG neurons were studied 16–24 h after dissociation to give the neurons a longer time to better withstand the repeated washings involved in the immunocytochemical measurement of NOS upregulation.

Axotomized neurons incubated with l-NAME, a specific inhibitor of NOS, had increased fast and the slow Na\(^+\) current densities compared with untreated axotomized neurons. NO may block Na\(^+\) currents through generation of cGMP (Moncada 1999) and/or through mechanisms independent of cGMP, i.e., via direct covalent interaction with susceptible thiol groups to form S-nitrosothiols that modify the protein function (Jia et al. 1996). Li et al. (1998) have reported that NO blocks Na\(^+\) currents in nodose ganglia via nitrosylation rather than through the generation of cGMP. However, whether the NO-mediated Na\(^+\) current block in small type C neurons is via cGMP and/or nitrosylation has not been determined.

The much smaller effect, if any, on the persistent TTX-R Na\(^+\) current on l-NAME incubation suggests that the channels that produce persistent current are present at very low levels in axotomized neurons. NaN transcript levels that are likely to encode persistent TTX-R channels (Cummins et al. 1999) are reduced significantly 7 days post axotomy in DRG neurons (Dib-Hajj et al. 1998; Tate et al. 1998). In our experiments, axotomized neurons were incubated with l-NAME for 12 h before the observed increase in fast TTX-S and slow TTX-R Na\(^+\) current density, a time period probably not sufficient enough to modulate the transcription and translation of channels. These results suggest that gene regulation rather than modulation of channels by NOS may account for most of the decrease observed in persistent TTX-R Na\(^+\) current in axotomized DRG neurons.

Consistent with a role of NOS in modulating the excitability of axotomized DRG neurons, Wiesenfeld-Hallin et al. (1993) have shown that NOS inhibitors suppress the ongoing discharges in DRG neurons after sciatic nerve injury. Our results show that exposure of axotomized neurons to l-NAME increased the number of cells expressing both fast Na\(^+\) current and slow Na\(^+\) current to 40% (from 21% in untreated cells) and increased the current density of fast and slow Na\(^+\) current density by 37 and 70%, respectively. These results suggest that NOS upregulation has effects on both fast TTX-S and slow TTX-R Na\(^+\) currents, possibly with a larger effect on slow Na\(^+\) current. At first sight, it might be expected that the downmodulation of both fast and slow Na\(^+\) current by NOS upregulation would decrease rather than augment DRG hyperexcitability. However, threshold and excitability of DRG neurons appear to be complex functions and depend on the densities of various types of Na\(^+\) channels (Cummins and Waxman 1997; Elliot 1997; Schild and Kunze 1997), possibly with optima at specific densities, above and below which excitability is altered. In SNS-null mutant mice, which do not express slow TTX-R Na\(^+\) channels but express fast TTX-S Na\(^+\) channels, compound action potentials recorded from the L4 dorsal roots had a lower electrical threshold and the recruitment curve was shifted significantly to the left (Akopian et al. 1999). TTX-R Na\(^+\) currents paradoxically might play a role in limiting the excitability of sensory neurons. The downmodulation of slow Na\(^+\) current to a larger extent than the fast Na\(^+\) current presents a possible scenario where the fast Na\(^+\) current, which has a rapid recovery from inactivation in axotomized DRG neurons (Cummins and Waxman 1997), might play a major role in determining the excitability of the axotomized neuron. Alternatively, NO also might affect excitability by altering K\(^+\) conductance.

In conclusion, the present results demonstrate NOS-related changes in fast and slow Na\(^+\) channel function in axotomized but not in normal C-type DRG neurons. These findings suggest that activity of endogenous NO/NO-related species due to the upregulation of NOS could block both fast and slow Na\(^+\) currents in axotomized DRG neurons. NOS thus appears to play an autocrine role in regulating the Na\(^+\) currents of small C-type DRG neurons after injury.

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NO REGULATES Na⁺ CURRENTS IN AXOTOMIZED DRG NEURONS


