

Nitric Oxide Reversibly Impairs Axonal Conduction in Guinea Pig Spinal Cord

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ABSTRACT

Increased expression of the inducible and neuronal isoforms of nitric oxide synthase (NOS), and elevated concentrations of nitric oxide (NO) metabolites, are present within the central nervous system (CNS) following neurotrauma and are implicated in the pathogenesis of the accompanying neurologic deficits. We tested the hypothesis that elevated extracellular concentrations of NO introduced by the donor Spermine NONOate, induce reversible axonal conduction deficits in neurons of the guinea pig spinal cord. The compound action potential (CAP) and compound membrane potential (CMP) of excised ventral cord white matter were recorded before, during, and after bathing the tissue (30 min) in varying concentrations (0.25–3.0 mM) of Spermine NONOate. The principal results were a rapid onset, dose-dependent, reduction in amplitude of the CAP ($p < 0.05$) accompanied by depolarization of the CMP during NO exposure. These effects were largely reversible on washout, at low concentration of the donor (0.5 mM), but were only partially reversed at higher concentrations. Changes in the electrophysiological properties were not evident when the donor had been *a priori* depleted of NO. The results extend previous reports that NO induces reversible axonal conduction deficits. They provide new evidence of dissociation of the effects of NO on CAP and CMP during washout, and after prolonged exposure to the donor. They add support to the emerging concept that immune-mediated axonal conduction failure contributes to reversible neurologic deficits following neurotrauma and aid in understanding clinical phenomena such as spinal shock and neurologic recovery.

Key words: axons; conduction; guinea pig; nitric oxide; spinal cord injury

INTRODUCTION

INCREASED EXPRESSION of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) is evident in immune cells, neurons, and glia within the central nervous system (CNS) immediately following spinal cord injury (SCI) and traumatic brain injury (TBI) (Ahn et al., 2004; Diaz-Ruiz et al., 2004; Kwak

et al., 2005; Matsuyama et al., 1998; Nakahara et al., 2002; Satake et al., 2000; Wada et al., 1998; Wu et al., 1994). Elevated concentrations of nitric oxide (NO) and its metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), are also present within the cerebrospinal fluid (CSF) following CNS trauma (Yamada et al., 1997). The high concentrations of NO, together with the increased synthesis of peroxynitrite (ONOO^-), by the reaction of NO with

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the superoxide anion (O_2^-), have been implicated in the pathogenesis of trauma-related neurologic deficits (Bao et al., 2002; Beckman et al., 1993). High intracellular concentrations of NO, for example, lead to axonal degeneration through excitotoxicity-mediated apoptosis (Liu et al., 1997) and the highly reactive oxidant ONOO⁻ can cause tissue damage through several different pathways (Gatti et al., 1994; Hogg et al., 1992; Radi et al., 1991). Elevated extracellular concentrations of NO have been shown to induce reversible axonal conduction failure in *in vivo* and *ex vivo* nerve preparations, and are thought to underlie the neurological dysfunction associated with neuroinflammation (Kapoor et al., 1999; Redford et al., 1997; Shrager et al., 1998). The exact mechanisms of NO-induced reversible axonal dysfunction are unknown but likely involve (i) cyclic guanosine monophosphate (cGMP)-independent mechanisms, such as S-nitrosylation, in which NO covalently reacts with susceptible thiol groups to form *n*-nitrosothiols that modify the conformation of gap junction and/or ion channel proteins, or (ii) disrupted mitochondrial respiration, with compromised axolemmal ionic exchange processes, and depolarization of the resting membrane potential (Li et al., 1998; Redford et al., 1997; Renganathan et al., 2002; Sato et al., 1995; Shrager et al., 1998).

In order to further investigate the putative role of NO-induced axonal conduction failure, we examined the effects of bathing excised strips of white matter from the ventral cord of guinea pigs with varying concentrations of the NO donor Spermine NONOate. A benefit of using this particular donor is its relatively long biological half-life ($t_{50} = 39$ min) and its relatively stable production of NO in aqueous solution (Keefer et al., 1996). The concentrations of donor investigated (0.25–3 mM) yield concentrations of NO that are physiologically and clinically relevant (0–42 μ M) (Kroncke et al., 1997; Laurent et al., 1996; Redford et al., 1997; Wood et al., 1994). Electrophysiological recordings of the compound action potential (CAP) and resting compound membrane potential (CMP) (Shi et al., 2000) were made using a double sucrose gap technique. The results reveal NO-mediated reversible, concentration-dependent changes in CAPs and provide new evidence of associated changes in CMP. They add support to an emerging concept of immune-mediated alterations in axonal ion channel conductances, that is, “channelopathy” (Koller et al., 1997; Rose, 1998; Waxman, 2001), or ion exchange mechanisms, compounding neurological deficits from axonopathy and myelinopathy during the neuroinflammatory events that follow neurotrauma (Davies et al., 2006). These observations of the mechanisms underlying reversible neurologic deficits may contribute to understanding of clinical phenomena such as spinal shock

(Dietz et al., 2004), and its resolution, or conversions from one level of neurologic impairment to another (Fisher et al., 2005; Kirshblum et al., 2004).

METHODS

Isolation of Spinal Cord Tissue

The surgical procedure for isolating the spinal cord tissue has been described previously (Shi et al., 2002). In brief, adult female guinea pigs of 350–500 g in body weight (Harlan, USA) were anesthetized prior to surgery (80 mg/kg ketamine hydrochloride, 0.8 mg/kg acepromazine maleate, and 12 mg/kg xylazine, *i.m.*). The animals were then perfused transcardially with 500 mL (15°C) of oxygenated Krebs’ solution (NaCl 124 mM, KCl 2 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 1.2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, sodium ascorbate 10 mM, equilibrated with 95% O_2 , 5% CO_2 to a pH of 7.2–7.4), and the vertebral column was excised rapidly. The spinal cord was carefully removed from the vertebrae and placed in cold Krebs’ solution. The cord was initially separated into two halves by midline sagittal division, and the ventral white matter was then isolated (Fig. 1A). White matter strips were maintained in continuously oxygenated Krebs’ solution for at least 1 h prior to mounting in the recording chamber to ensure recovery from dissection before experimentation.

The studies were conducted using identical protocols and instrumentation at both the Institute for Applied Neurology, Purdue University, West Lafayette, Indiana, and the Lawson Health Research Institute/University of Western Ontario, London, Ontario, Canada. The experimental protocols were reviewed and approved by the Purdue University Animal Care and Use Committee and the University of Western Ontario Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their distress.

Electrophysiological Recording and Analysis

Electrophysiological recordings from the isolated cord tissue were made using a double sucrose gap chamber. The design and construction of the chamber has been described previously (Shi et al., 1996) and is illustrated in Figure 1B. The strips of spinal cord white matter, 38–45 mm in length and ~ 1.5 mm in diameter, were positioned across the three compartments of the recording chamber. The central compartment, 20 mm in diameter, was continuously superfused with oxygenated Krebs’ solution at 2 mL/min into which the Spermine NONOate was introduced. The temperature of the central compartment was maintained at 37.0°C with an in-line solution warmer

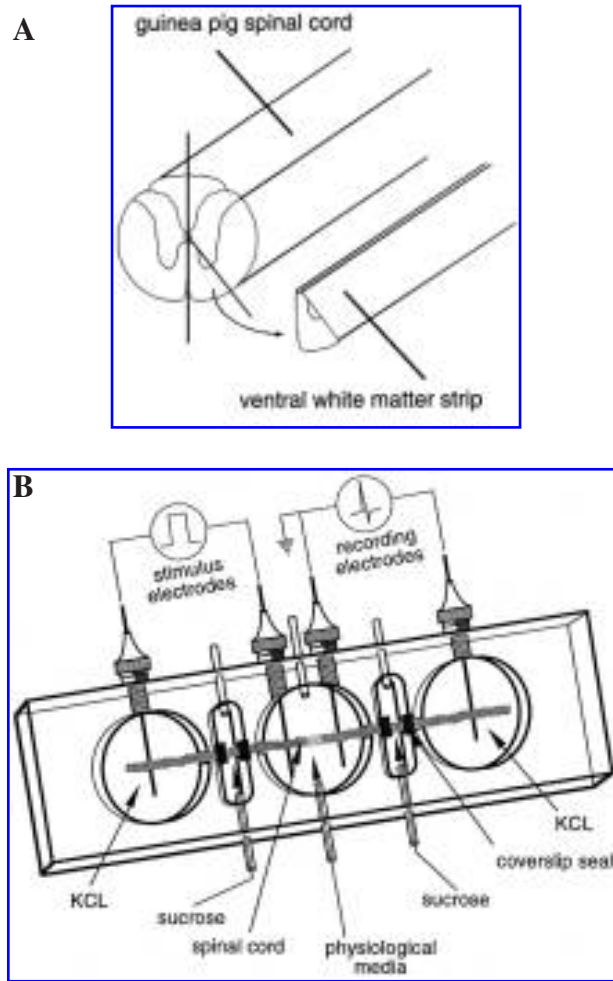


FIG. 1. Isolation of ventral white matter and double sucrose gap chamber for *ex vivo* recording of axonal conduction properties of excised guinea pig ventral white matter. **(A)** Surgical isolation of ventral white matter strips for mounting in the double sucrose gap recording chamber. **(B)** Double sucrose gap recording chamber viewed from above showing the two outside compartments filled with isotonic KCl, a central compartment filled with oxygenated Krebs' solution, and two sucrose gap compartments maintaining electrical isolation between KCl and Krebs'. Nitric oxide (NO) donor Spermine NONOate is added to the Krebs' solution to bathe the cord in the central compartment. The guinea pig cord is positioned in the trough that traverses the various compartments and is covered by the fluids. Stimulation electrodes (silver-silver chloride) with anode and cathode positioned in the KCl and Krebs' compartments, and bipolar recording (pin) electrodes in the opposite KCl compartment and the central Krebs' compartment are also shown. The central recording electrode is referenced to ground.

(Warner Instruments, Hamden, CT), and monitored using a thermocouple probe. The two end compartments contained isotonic potassium chloride (120 mM) and were electrically isolated from the central compartment

by narrow channels through which sucrose solution (320 mM) flowed continuously.

Each strip of tissue was stimulated through silver-silver chloride electrodes positioned with one in a side compartment and the other in the central bath (Fig. 1B). Constant current stimuli (supramaximal) were generated by a digital stimulator (Cygnus Technology, Delaware Water Gap, PA) and delivered via a stimulus isolation unit (WP Instruments, Sarasota, FL) in the form of 0.1 msec constant current rectangular pulses at a rate of 0.33 Hz. The evoked CAPs, formed by the spatio-temporal summation of many single action potentials, were recorded continuously throughout the study. The monophasic CAP waveform was recorded from silver, silver-chloride pin electrodes positioned in the central (grounded) and end chambers. The digitized (25 kHz) profile of each CAP was recorded for subsequent processing (e.g., peak amplitude, averaging); in addition a real time plot of CAP amplitude was displayed during the experiment and saved for further analysis. The potential across the recording electrodes on either side of the sucrose gap was also measured just prior to each stimulation. This potential, sometimes referred to as the DC potential or "gap potential" (Shi et al., 2002), is proportional to the resting compound membrane potential (CMP) and this latter term is used in this report. The changes in this potential were expressed as a percentage of the maximum depolarization brought about by exposure to the NO donor. In some preparations, the cord was transected in the central chamber at the end of the study in order to obtain estimates of the total voltage change associated with complete depolarization. All data were digitized and stored with a Neurodata Instruments Neurocorder for subsequent analysis. The on-line recording and subsequent analysis was performed using LabView software (National Instruments, Austin, TX).

Nitric Oxide Treatments

The NO donor, Spermine NONOate (AXXORA, San Diego, CA), with $\geq 98\%$ purity, was added to oxygenated Krebs' solution (pH 7.4) in concentrations of 0.25, 0.5, 1.0, 2.0, and 3.0 mM. The Krebs' solution was maintained at 37°C. Under these conditions, the donor is relatively stable, having a half-life (t_{50}) of 39 min (Keefer et al., 1996), and its NO release kinetics have been well characterized (Maragos et al., 1991). In Appendix A, we summarize the mathematical model developed by Schmidt et al. (1997), which we used to estimate extracellular NO concentrations in aerobic, aqueous solution, under the present conditions, achieved by decomposition of Spermine NONOate. The model has been well validated against experimental measurement of NO concentrations and was devel-

oped because of the technically complex methods required for *in situ* measurement. These calculations were used to estimate the extracellular concentration of NO that the tissue was exposed to during the 30-min experimental treatment and in a control condition when the tissue was exposed to Spermine NONOate for a period of 5 h.

Fresh strips of tissue (five replications) were used to study the effects of each concentration of Spermine NONOate. The duration of perfusion with Spermine NONOate was 30 min. After 30 min, the preparation was perfused with Krebs' solution alone in order to wash out the NO donor (and NO), and the electrophysiological properties of the preparation were monitored for an additional 60 min.

Control Condition

Four control conditions were employed. In the first, a different Krebs' solution was introduced in lieu of Spermine NONOate. In the second, 2 mM of Spermine NONOate was added to the Krebs' solution 5 h prior to bathing the cord tissue (i.e., >7 times the half-life of the donor). This enabled evaluation of the effects of the NO depleted donor. NO itself has a half-life of less than 5 sec in aqueous solution (Hakim et al., 1996; Wink et al., 1993); the time course of dissociation of NO therefore closely approximates the degradation of the donor ($t_{50} = 39$ min). The third control involved longer term (5 h) of exposure of the tissue to Spermine NONOate (2 mM) to establish the correspondence, if any, between electrophysiological changes and the predicted kinetics of NO dissociation (allowing for donor degradation). A fourth control enabled recording of the electrophysiological properties of the preparation, in the absence of Spermine NONOate, also for an extended period of time (5 h), to establish the long term stability of the CAP and CMP measures.

Statistical Analysis

The mean CAP waveform recorded from each preparation before, during, and after treatment with varying concentrations of Spermine NONOate was obtained by averaging the digitized signal from 50 individual CAPs. The CAP amplitude was expressed as a percentage of the baseline mean CAP amplitude for each preparation. The changes in CMP were expressed as a percentage of the maximum Spermine-induced depolarization. Least squares linear and sigmoid regression algorithms were used to characterize the association between NO donor concentration and CAP amplitude during NO exposure and washout, respectively.

RESULTS

Stabilization

The CMP stabilized over a mean 51.15 ± 9.0 (SD) min in the 38 preparations (15 animals) tested. During this time, the CMP recorded across the sucrose gap became increasingly polarized, most likely a reflection of decreased leakage current across the gap and increasing resistance of extracellular pathways as a result of sucrose perfusion (Shi et al., 1996). Once stabilized, the CMP varied minimally (0.01–0.06 mV; SEM) prior to introduction of NO. Monophasic CAPs (1–5-mV amplitude) were recorded from each preparation. An illustrative set of CAPs from a single preparation exposed to Spermine NONOate (3 mM) is shown in Figure 2, together with the accompanying changes in CMP.

Effects of Varying Concentrations of Spermine NONOate on Compound Action Potential and Compound Membrane Potential

Application of Spermine NONOate at a concentration of 0.25 mM ($n = 5$) had no effect on the CAP or CMP throughout the duration of the exposure phase (30 min) and the subsequent washout period (60 min). A concentration of 0.5 mM Spermine NONOate resulted in a slow and persistent partial depolarization of the CMP in all preparations ($n = 5$). The depolarization started within minutes of exposure in all preparations and progressed for 20–30 min. The maximum depolarization for each preparation was designated 100% to allow comparison across preparations. Concurrently, there was a progressive reduction in CAP amplitude. The minimum CAP amplitude occurred at close to 30 min of exposure to Spermine NONOate, and the mean CAP amplitude was reduced from 1.69 ± 0.8 mV, to 0.82 ± 0.2 mV, or 48.5% of pre-treatment CAP amplitude. Following 60 min of washout with oxygenated Krebs' solution, the mean CAP amplitude returned to $91.34 \pm 8.4\%$ of pre-treatment levels, and the CMP repolarized, but in each case failed to reach pre-treatment values. The changes in mean CAP amplitude, across all preparations ($n = 5$), with varying concentrations of Spermine NONOate are illustrated in Figure 3A.

Application of Spermine NONOate at concentrations of 1, 2, and 3 mM all resulted in an early onset and prolonged partial depolarization of the CMP in each one of the preparations ($n = 5$). At these concentrations, the depolarization reversed from 100% to 60–40% of pre-treatment values after 60 min washout (i.e., the CMP was only partially repolarized when the Spermine NONOate was removed).

At each of the 1, 2, and 3 mM concentrations, the CAP amplitude reduced within minutes of exposure to Sper-

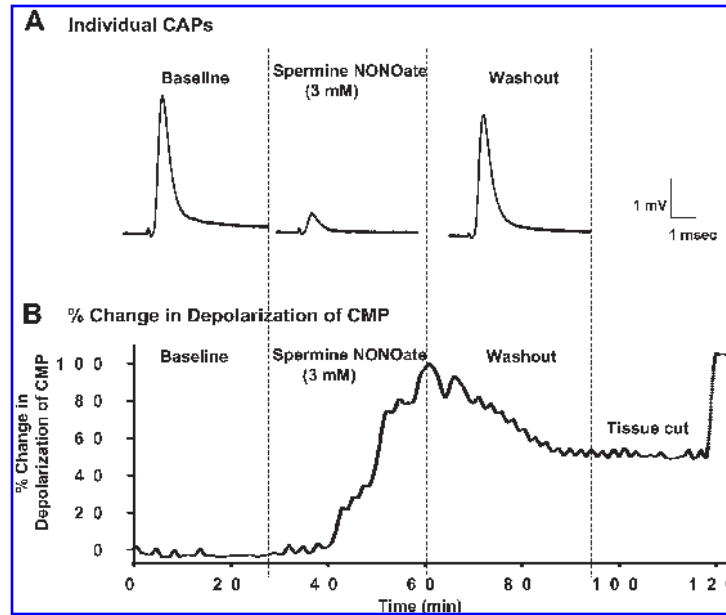


FIG. 2. Changes in compound action potential (CAP) waveforms and compound membrane potential (CMP) before, during, and after treatment with 3 mM Spermine NONOate. **(A)** Averaged ($n = 50$) CAP waveforms recorded from one preparation before, during, and after treatment with 3 mM Spermine NONOate. The nitric oxide (NO) donor caused a reduction in CAP amplitude that was largely reversed on washout. The duration of each trace is 7 msec. **(B)** CMP waveform from the same preparation recorded before, during, and after treatment with 3 mM Spermine NONOate. The NO donor caused profound depolarization of the CMP which was partially repolarized on washout.

mine NONOate and continued to reduce for 20–30 min. At 1 mM the minimum CAP amplitude occurred after 30 min exposure to the NO donor, at which time the mean CAP amplitude was reduced to $27.9 \pm 4.5\%$ of its pre-treatment value (1.52 ± 0.6 mV to 0.42 ± 0.4 mV). Following 60 min of washout with oxygenated Krebs' solution, the mean CAP amplitude had recovered to 96.4% of pre-treatment levels (1.46 ± 0.6 mV). Mean CAP amplitude (percent) from all five preparations exposed to 1.0 mM Spermine NONOate are illustrated in Figure 3A (open circles). At the 2 mM concentration, the minimum CAP amplitude occurred after 26.4 min of exposure to NO, and the mean CAP amplitude was reduced to $14.9 \pm 9.9\%$ of the pre-treatment value. Sixty minutes of washout with oxygenated Krebs' solution reversed the reduction in amplitude; the CAP amplitude recovered to $91.1 \pm 13.9\%$ of the pre-treatment value. The changes in mean CAP amplitude for five preparations are shown in Figure 3A (filled inverted triangles). At the 3 mM concentration, the CAP amplitude dropped precipitously on introduction of NO and in all preparations reduced to $<5\%$ of the pre-treatment values. The minimum CAP amplitude occurred at 2–5 min of exposure to Spermine NONOate, and the mean CAP amplitude was reduced from 2.34 ± 0.77 to 0.10 ± 0.097 mV or 4.3% of pre-

treatment baseline value. Sixty minutes of washout with oxygenated Krebs' solution reversed the decrease in CAP amplitude and it recovered to $94.3 \pm 2.5\%$ of the pre-treatment value. Mean CAP amplitude values for five preparations exposed to 3 mM Spermine NONOate are illustrated in Figure 3A (open triangles).

Concentration-Dependent Conduction Failure

The concentration-dependent nature of the NO-induced conduction failure across all preparations is illustrated in Figure 3B. The normalized peak CAP amplitude (y) reduced exponentially with the NO treatment concentration ($y = 1.78 \cdot (1/2.45)^{[Spermine\ NONOate]}$), $R^2 = 0.95$, $p < 0.01$). As treatment concentration increased, reductions in CAP amplitude and depolarization of the CMP occurred progressively sooner following NO exposure. These changes in CAP are evident in Figure 3B.

Temporal Relationship between Spermine-Induced Compound Action Potential and Compound Membrane Potential Changes

The temporal associations between changes in CAP amplitude and CMP, for individual preparations, at four different concentrations of Spermine NONOate, are

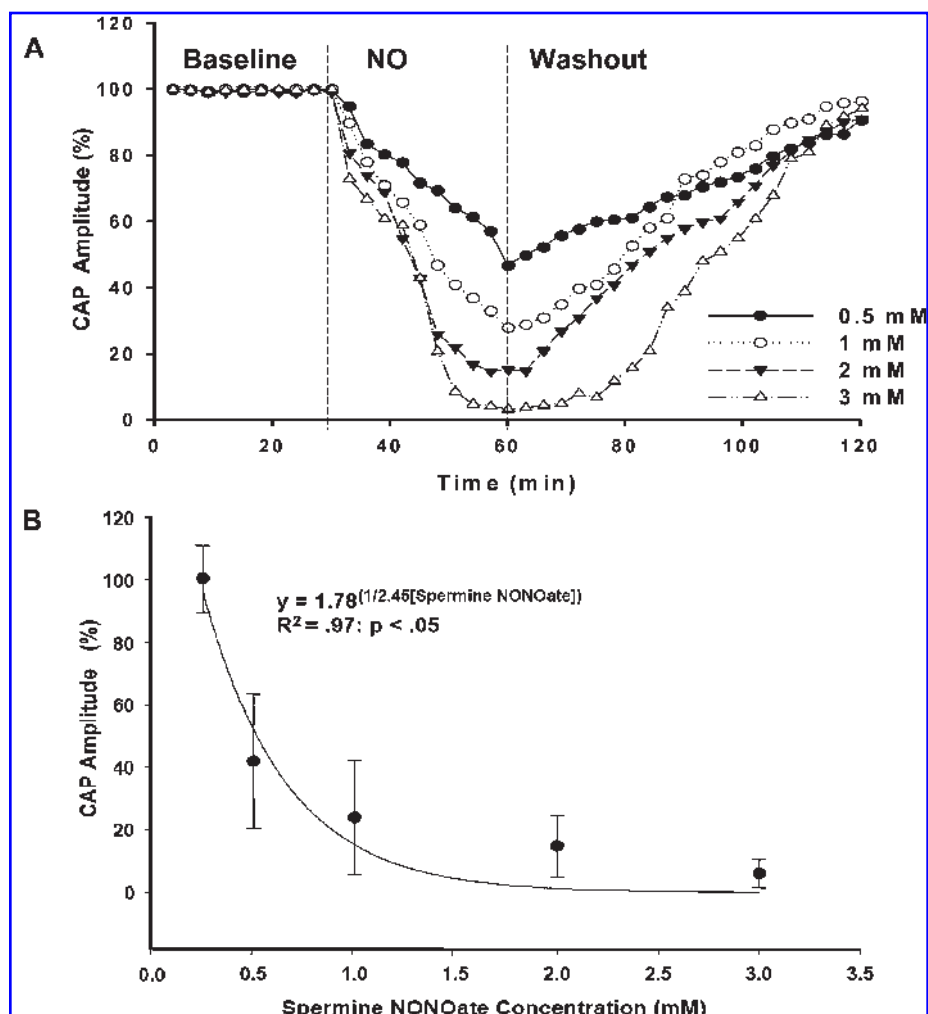


FIG. 3. (A) Effects of varying concentrations of Spermine NONOate on compound action potentials (CAPs) in different preparations. Application of 0.5 mM Spermine NONOate (filled circles) resulted in a progressive reduction in CAP amplitude; following 60 min of washout with oxygenated Krebs' solution the CAP amplitude recovered to 100% of baseline values. Following treatment with 1 mM Spermine NONOate (open circles), CAP amplitude reduced within minutes of exposure and continued to reduce for 15–25 min. Following washout, CAP amplitude recovered to baseline values. Spermine NONOate at a concentration of 2 mM (filled inverted triangles) resulted in a rapid reduction in the CAP amplitude. In some preparations no CAP was recorded. The minimum CAP amplitude occurred after 26 min of exposure to nitric oxide (NO). The 60 min of washout with oxygenated Krebs' solution reversed the reduction in amplitude. Application of Spermine NONOate at a concentration of 3 mM (open up-right triangles) resulted in precipitous drop of the CAP amplitude. The minimum CAP amplitude occurred at 2–5 min of exposure to Spermine NONOate. The 60 min of washout with oxygenated Krebs' solution largely reversed the decrease in CAP amplitude, and it recovered to close to pre-treatment levels. (B) Concentration-dependency of NO-induced conduction block. The normalized peak CAP amplitude (y) reduced exponentially with the NO treatment concentration ($y = 1.78 \cdot (1/2.45^{[Spermine\ NONOate]})$, $R^2 = 0.95$, $p < 0.05$). As treatment concentrations increased, reductions in CAP amplitude occurred following NO exposure. The vertical bars represent standard deviations across the five preparations studied at each concentration.

shown in Figure 4. There was generally a close correspondence between the reduction in CAP amplitude and CMP depolarization during the exposure to Spermine NONOate. However, during washout, the CAP recovered toward its baseline value at a faster rate and to a greater

extent than did the CMP. This difference in rate and extent of recovery was evident in all preparations and at all concentrations. Figure 5 summarizes the collective data for this effect when five preparations when exposed to 1 or 3 mM concentrations of the NO donor. There was a

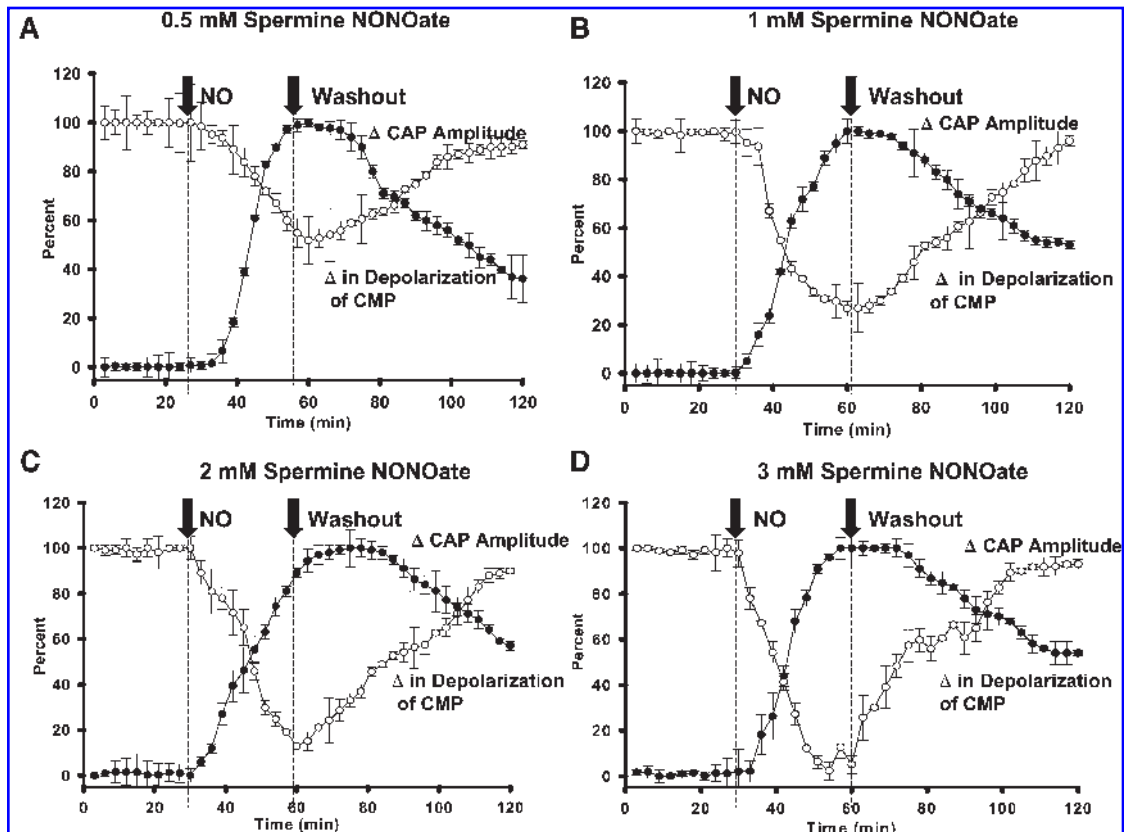


FIG. 4. Temporal relationship between Spermium-induced compound action potential (CAP) and compound membrane potential (CMP) changes. The temporal associations between changes in CAP amplitude (open circles) and CMP (filled circles), for individual preparations, at four different concentrations of Spermium NONOate, are illustrated. Individual preparations treated with 0.5 mM (A), 1 mM (B), 2 mM (C), and 3 mM (D). Spermium NONOate resulted in a generally close correspondence between the reduction in CAP amplitude and CMP depolarization during the exposure to Spermium NONOate. During washout, the CAP recovered toward its baseline value at a faster rate, and to a greater extent, than did the CMP. This difference in rate and extent of recovery was evident in all preparations and at all concentrations. Note the change in CMP has been normalized to 100% of the maximum amount of depolarization. Vertical error bars represent standard deviations; CAPs were averaged every 5 min.

linear relationship ($R^2 = 0.99$ for 1 mM and 0.98 for 3 mM) between CAP and CMP changes during exposure to Spermium NONOate and a sigmoidal relationship ($R^2 = 0.98$ for 1 mM, and 0.99 for 3 mM) during washout from both concentrations. These differences in the reversal of CAP and CMP changes during washout suggest differential effects of NO on the electrophysiological mechanisms (ion channels and ionic pumps) subserving the CAP and CMP, respectively.

Nitric Oxide Release Kinetics and Electrophysiological Changes

The NO release kinetics of Spermium NONOate predicted by the Schmidt et al. (1997) model for the five concentrations and the specific experimental conditions employed here are shown in Figure 6. The NO released

varied from an initial (5 min) concentration of 42 μM from 3 mM Spermium NONOate to 2.6 μM from 0.25 mM of the donor. The instantaneous rate of NO release depends on the degradation rate of the donor and the concentration of NO drops by $\sim 29\%$ during the course of 30-min exposure for each concentration of donor. Examination of the time course of CAP and CMP changes in relation to NO concentration for the control preparation exposed to 2 mM for 300 min without washout shows an essentially linear relationship during the first 30 min of exposure. Both CAP and CMP changes were similar to those seen in the 30-min experimental condition (albeit somewhat time lagged for reasons that are not clear). Figure 6B,D shows that there was a pronounced reduction in CAP amplitude for the first 100 min when the NO concentrations were greater than $\sim 3 \mu\text{M}$. This is shown at the vertical broken line (i) in Figure 6B. When the NO

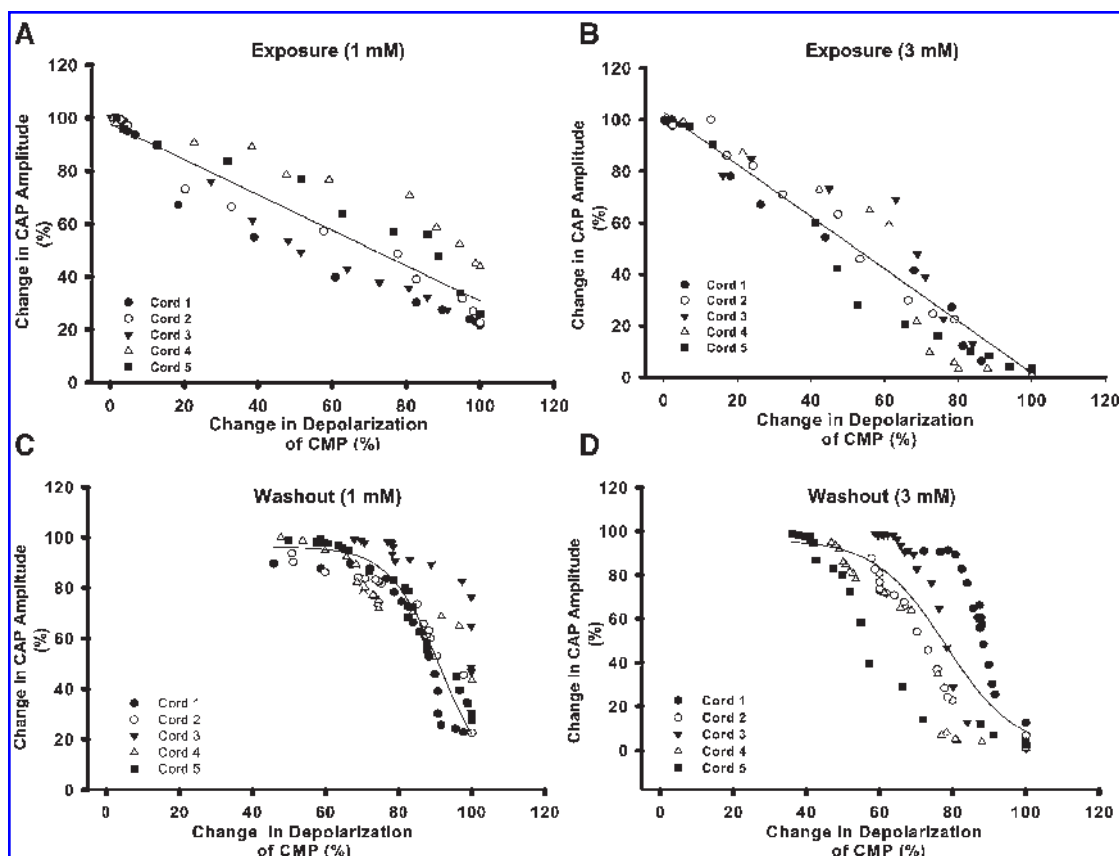


FIG. 5. Linear relationship between compound action potential (CAP) and compound membrane potential (CMP) changes during exposure to Spermine NONOate and a sigmoidal relationship during the washout phase. (A) Five preparations exposed to 1 mM of Spermine NONOate yielded a linear relationship ($R^2 = 0.99$) between CAP and CMP changes during the exposure phase and a sigmoidal relationship ($R^2 = 0.98$) during the washout phase (C). (B) Five preparations treated with 3 mM Spermine NONOate also resulted in a linear relationship between CAP and CMP changes during the exposure phase ($R^2 = 0.98$) and a sigmoidal relationship ($R^2 = 0.99$) during the washout phase (D). These graphs illustrate the differential effects of nitric oxide (NO) on CAP and CMP during washout.

release rate dropped below this value, the CAP amplitude recovered (albeit not to 100% as also occurred in the 30-min exposure without washout condition). The time course of CMP changes differed from the CAP changes in as much as the repolarization did not start until 140–150 min when $\sim 2 \mu\text{M}$ (vertical broken line ii). The CAP stabilized after 160–170 min whereas the CMP continued to repolarize from ~ 150 min to 215 min. NO concentration was less than $4 \mu\text{M}$ at ~ 125 min.

Control Conditions

Replication of the 30-min treatment protocol, but using replacement Krebs' solution (Control 1) or NO-depleted donor (Control 2), yielded no changes in peak CAP amplitude or depolarization of CMP. Control condition 3, in which no Spermine was introduced but CAPs and CMP were monitored for 5 h, also revealed no change in elec-

trophysiological properties. The stability of the peak amplitude of the CAP recordings over this time frame is evident in Figure 6C. Finally, the control condition 4, in which the tissue was exposed to 2 mM Spermine NONOate for 5 h and there was no washout, and CAP and CMP recordings were made continuously, not only enabled comparison of the time course of electrophysiological changes with predicted NO concentrations (Fig. 6B,D) but independently confirmed the differences in recovery properties of CAP and CMP as NO was eliminated (cf. Fig. 4).

DISCUSSION

Immune-mediated axonal conduction deficits are emerging as important considerations in the pathogenesis of neurologic deficits arising from various central neu-

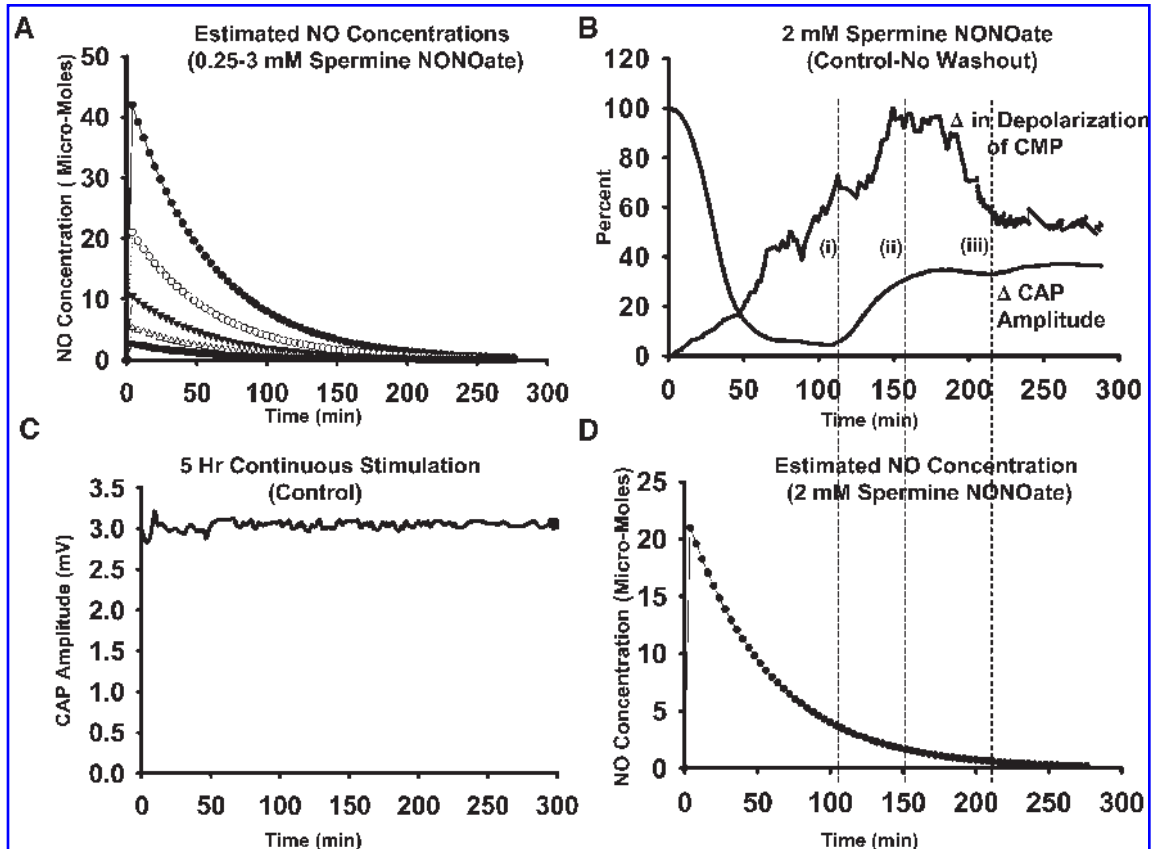


FIG. 6. Nitric oxide (NO) release kinetics and electrophysiological changes. (A) NO release kinetics of Spermine NONOate predicted by the Schmidt et al (1997) model for five different concentrations. The NO release varied from an initial (5 min) concentration of $42 \mu\text{M}$ from 3 mM Spermine NONOate to $2.6 \mu\text{M}$ from 0.25 mM of donor. The concentration of NO dropped by $\sim 29\%$ during the course of the 30-min exposure for each concentration of donor. (B,D) Time course of compound action potential (CAP) and compound membrane potential (CMP) changes in relation to NO concentration for the control preparation exposed to 2 mM Spermine NONOate for 300 min; comparisons of (B) and (D) show that there was a pronounced reduction of CAP amplitude at NO concentrations greater than $3 \mu\text{M}$ (~ 100 min exposure, i.e., vertical broken line i). When the NO release rate dropped below this value, the CAP amplitude recovered partially. The time course of CMP changes differed from the CAP changes inasmuch as the repolarization did not start until NO concentration was less than $1.5 \mu\text{M}$ at ~ 150 min: vertical broken line ii. The CMP remained at about 60% of its pre-treatment level when NO release had reached zero (vertical broken line iii). (C) CAP amplitude recorded during control condition; no Spermine NONOate was introduced but CAPs were monitored continuously for 5 h and revealed no systematic change in peak amplitude.

roinflammatory diseases and neurotrauma (Brown et al., 2003; Calabrese et al., 2000; Garthwaite et al., 2002; Köller et al., 1997). NO, a free radical synthesized from L-arginine by the three independent isoforms of NOS, is an immune-mediator that has been implicated in the neurologic dysfunction associated with multiple sclerosis (Rejdak et al., 2004). The exact relationship between elevated levels of NO metabolites in CSF and disease activity remains in dispute, however, as does the concentration of NO or its metabolites necessary to cause cell damage (Garthwaite et al., 2002). Indeed, as with other immune mediators, elevated concentration of NO may

also have beneficial properties and the actual physiological consequences at any specific time are likely dependent on other concurrent immunochemical processes (Iadecola, 1997).

Immediately following SCI, when there is a profound neuroinflammatory response, there is a rapid and transient increase in the expression of constitutive NOS in endothelial (eNOS) and neuronal cells (nNOS), respectively (Isaksson et al., 2005). This is followed by increased expression of iNOS in macrophages and glial cells, resulting in elevated production of NO, which can lead to free radical-induced tissue injury. Specifically

NO, or its byproduct peroxynitrite, has been implicated in cytokine-mediated cytotoxicity of neurons and oligodendrocytes (Brown et al., 2003), and in anoxia-induced axonal necrosis and apoptosis following both TBI and SCI (Liu et al., 1997; Lu et al., 2003). Various immunomodulatory therapeutic strategies are currently being explored to mitigate the injurious consequences of elevated intra and extracellular concentrations of NO or its byproducts and to confer neuroprotection (Chatzipanteli et al., 2002).

In the present study, we examined the axonal conduction blocking effects of NO, delivered by various concentrations of the NO donor Spermine NONOate, using excised ventral cord white matter from guinea pigs. Previous studies have shown that relatively low extracellular concentrations of NO induce reversible conduction failure in both central and peripheral axons (Kapoor et al., 1999; Redford et al., 1997; Shrager et al., 1998). These observations raise the possibility that some of the reversible neurologic deficits seen following CNS trauma may be attributable to resolution of NO-mediated conduction abnormalities. Unlike previous studies of the effects of NO on central axonal conduction, in which NO donors were injected into the dorsal column of rats, we elected to bathe the tissue for 30 min, *ex vivo*, in varying concentrations of Spermine NONOate while the electrophysiological properties were monitored. The more prolonged exposure of the present study was thought to represent conditions more relevant to the *in vivo* clinical state where elevated iNOS levels persist (Simmons et al., 1992; Zielasek et al., 1992) and to avoid any inflammatory response induced by injection *per se*.

The principal results of the present study were a rapid-onset, dose-dependent, depolarization of the CMP temporally associated with a reduction in CAP amplitude, during the initial exposure to NO. This was evident at donor concentrations greater than 0.5 mM when the predicted concentration of NO *per se* in Krebs' solution was $>4 \mu\text{M}$. The rate of onset of these electrophysiological changes increased with the concentration of NO. The effects on the CAP were reversible on washout of donor, at all donor concentrations above 0.5 mM, and were not present when the delivery medium, Krebs' solution, was administered alone or when the Spermine NONOate was depleted of its NO. In contrast, the effects of NO exposure on the CMP were not fully reversible on washout and indeed followed a different time course (or concentration-dependence) from the CAP changes when the tissue was left exposed for a prolonged (5-h) period. The physiological mechanisms underlying these NO-mediated electrophysiological changes remain to be determined; however, the rapidly reversible nature of the CAP changes suggests that the impairment of axonal conduction was not due to axonopathy or oligodendroglialopathy

per se. Previous studies of NO-mediated alterations in neuronal activity have eliminated cGMP dependent mechanism as being contributory (Li et al., 1998; Shrager et al., 1998). Reversible alterations in the conductance of Na^+ , K^+ , Ca^{2+} , and non-selective cation or *N*-methyl-D-aspartate receptor/channel complexes, or disruption of the ionic exchange pumps necessary for maintaining and restoring membrane potential therefore emerge as likely mechanisms (Li et al., 1998; Redford et al., 1997; Renganathan et al., 2002; Sato et al., 1995; Shrager et al., 1998).

Shrager et al. (1998) demonstrated NO-mediated reversible axonal conduction failure in rat peripheral nerve using the donor diethylamine-NONOate. Based on the rapidity, thoroughness and reversibility of NO block of CAPs, they suggested that the conduction failure might be attributable to an effect of NO on voltage gated Na^+ channels. In order to determine if NO-induced conduction failure was mediated by cGMP (a second messenger, released by the NO receptor soluble guanylate cyclase [sGC]), they exposed cultured neurons to the membrane-permeable cGMP analog 8-bromo-cGMP. 8-Bromo-cGMP (2 mM) did not yield conduction block comparable to that induced by NO donors, suggesting a cGMP-independent mechanism of NO-induced conduction failure. They further postulated that the formation of a nitrosothiol, through reaction of NO with a sulfhydryl group (S-nitrosothiol), might lead to inactivation of Na^+ channels, as has been shown previously in invertebrate axons (Shrager, 1977; Starkus et al., 1978). Li et al. (1998) have further established that exogenous NO donors inhibit both tetrodotoxin-sensitive and insensitive Na^+ currents through NO interaction with channel thiols. S-nitrosothiol is formed when thiols (S – X) react with either NO or ONOO^- to form disulfide bonds. Sulfhydryl reactive compounds of this form are capable of altering the conformation of proteins that form gap junction channels, eventually causing them to either remain open or closed (Campos de Carvalho et al., 1986; Renganathan et al., 2002). Renganathan et al. (2002) subsequently demonstrated that NO blocks fast, slow, and persistent Na^+ channels in C-type dorsal root ganglion neurons of rat by S-nitrosylation. The half-life of S-nitrosothiols range from minutes to hours and this may explain the reversibility of their effects on axonal conduction.

NO-mediated alteration in mitochondrial ATP production within axons is another mechanism by which NO can potentially depolarize the axonal cell membrane and attenuate CAP amplitude. Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain and is responsible for $>90\%$ of cellular oxygen consumption. The enzyme catalyses the oxidation of cy-

tochrome C^{2+} to cytochrome C^{3+} and the reduction of oxygen to water, which is coupled to the pumping of protons across the inner mitochondrial membrane (Stys et al., 1992). Since NO or ONOO⁻ competes with oxygen at the cytochrome c oxidase, increases in intracellular NO and/or ONOO⁻ concentration can prevent the enzyme from using any available oxygen, causing a kind of "metabolic hypoxia" (Moncada et al., 2002). Thus, NO may disrupt mitochondrial ATP production leading to failure of the Na⁺/K⁺ ATPase and reverse operation of the Na⁺/Ca²⁺ exchanger (Stys et al., 1992, 1998). These effects, in turn, can therefore lead, initially, to over accumulation of intracellular Na⁺ and eventually to over accumulation of Ca²⁺, potentially to cytotoxic levels. Excess accumulation of axoplasmic Na⁺ per se will induce membrane depolarization sufficient to cause conduction failure. Rapid depletion of ATP and failure of Na⁺/K⁺ ATPase may thus account for the pronounced but reversible membrane depolarization caused by NO treatments at low concentrations in the present study. If the intracellular Na⁺ concentration is sufficiently elevated or maintained for extended period of time, this leads to elevated intracellular Ca²⁺ concentrations, which elevate constitutive NOS (eNOS and nNOS) activity, resulting in even greater synthesis and release of NO. The elevated Ca²⁺ concentration may become cytotoxic and lead to irreversible changes in membrane properties.

The outcomes of the present study do suggest the involvement of two different processes by which NO alters the electrophysiological properties of axons within the CNS. The first process, causing depolarization of the cell membrane and reduction in amplitude of the CAP during exposure to relatively low (but still elevated) concentrations of NO (>4 μ M), is fully reversible on washout of the NO donor. The immediacy of onset (i.e., with only brief exposure) and full reversibility might suggest the direct involvement of altered conductance in ion channels that are involved in maintenance of the resting potential and genesis of the CAP. The second process, which may come into play at low concentrations but has more prominent effects at higher concentrations of NO, or with prolonged exposure, eventually causes irreversible changes in the CMP at a time when CAP changes are still largely reversible. These effects are still present when NO concentrations have reduced to very low levels because of degradation (or washout) of the donor. The properties of these two different processes bear some resemblance to those which would be expected to accompany S-nitrosothiol-mediated block of Na⁺ channel conductance and NO-mediated disruption of mitochondrial respiration respectively. The ion exchange processes involved in generation of the CAP are independent of ATP whereas the pumps and ion exchange processes subserv-

ing the ionic equilibrium of the CMP are ATP-dependent. Clearly more targeted mechanistic studies are required to test this speculation and identify the precise mechanisms involved.

In order to properly evaluate the physiological relevance of the present results it is necessary to give careful consideration to the experimental concentrations of NO donor employed (0.25–3.0 mM), the estimated concentrations of extracellular NO induced (0–42 μ M), and the *in vivo* concentrations of NO present under normal and pathological states. Physiological concentrations (extracellular) of NO are 0.01–1 μ M *in vivo* (Hakim et al., 1996). Following neurotrauma, there are multiple additional cellular sources of NO. NO concentration in a single activated macrophage *in vitro* reaches 1 μ M in approximately 10 sec, and production can continue for many hours (Shrager et al., 1998). The abundance of macrophages and other NO producing immune cells at the site of injury (Hayes et al., 1997; Wang et al., 2000) would provide multiple sources of NO, which would summate and eventually reach steady state concentration (Kroncke et al., 1997). A steady state of about 4–5 μ M of NO in the immediate vicinity of the target cell monolayer has been reported in *in vitro* models (Laurent et al., 1996). The diffusion distance of NO secreted by a single cell is large (150–300 μ m in 4–15 sec) and is further enhanced when cells are located in clusters (Lancaster, 1996). Activated macrophages at target cells are capable of producing 15–40 μ M of NO within a 15-h timeframe of activation (Kroncke et al., 1997; Steiner et al., 1997). In order to mimic these concentrations in cell culture using NO donors, concentrations of 0.5–1 mM of the donors are necessary. These concentrations yield cytotoxic outcomes when prolonged exposure times are employed (Kroncke et al., 1997). Most of the NO generated via chemical sources (i.e., donors) is probably autooxidized prior to reaching target cells whereas the cellular sources of NO production (i.e., macrophages and glia) produce NO in close proximity to target cells, thus allowing little opportunity for autooxidation. Therefore, *in vitro* studies in general are likely to underestimate the physiological and toxicity effects of NO *in vivo*.

It has been established elsewhere, using assays of the NO receptor enzyme sGC, that the concentrations of donors used in the tissue bathing model are necessarily many times greater than those required at the *in vivo* local interstitial level to induce comparable effects (Garthwaite et al., 2002). The estimated values of extracellular NO concentration at which reversible changes in electrophysiological properties occurred in the present study (0–42 μ M) thus appear to be relevant and generally comparable to those likely present during the immune-mediated neuroinflammation that occurs following CNS

trauma. It is also important to acknowledge that it is not the extracellular concentration of NO alone that is important but the combined effect of concentration and duration of exposure that is critical in determining the degree of neuromodulatory influence or cytotoxicity (Takeuchi et al., 1998; Trudrung et al., 2000).

The clinical relevance of the present evidence of NO-mediated conduction failure resides in its potential ability for explaining functional deficits that are disproportionate to frank neurological damage following CNS trauma.

There are diverse manifestations of functional neurological deficits that exceed what would be expected given the degree of tissue sparing following trauma. In the acute stage following trauma, when neuroinflammation is evident and both intracellular and extracellular NO concentrations are elevated, NO-mediated conduction deficits may contribute to phenomena such as “spinal shock” (Ditunno et al., 2004) or levels of neurological impairment that convert (i.e., resolve spontaneously) (Fisher et al., 2005; Kirshblum et al., 2004) or as the cytokine “challenge” and neuroinflammation resolve. If, as previously speculated, a chronic neuroinflammatory process is brought about through elevated levels of circulatory proinflammatory cytokines crossing the blood–brain barrier, then increased cytokine-induced NO production would be expected to lead to long-term neurologic deficit, and resolution would lead to late onset neurologic recovery (Davies et al., 2006; Hayes et al., 2002). More generally, however, the present results do support the proposition of immune-mediated conduction deficits brought about by modified ion channel conductance (i.e., acquired “channelopathy” or ion pump function), contributing to the neurological outcome following SCI and other forms of neurotrauma (e.g., peripheral nerve injury) (Waxman, 2001). This mechanism of deficit may provide a target for restorative immunomodulatory therapies.

In summary, the present results yield new evidence that NO causes a concentration-dependent reversible conduction block in axons of the spinal cord. Increasing concentrations of the NO donor Spermine NONOate yielded progressively greater reductions in amplitude of the CAP accompanied by depolarization of the CMP. The conduction blocking effects were reversed on washout and were not present when the NO donor was completely depleted of NO. Since elevated levels of NO and its derivatives are present within the CNS of patients following neurotrauma, it seems plausible that NO-mediated impairment of axonal conduction may underlie some of the associated neurological deficits, and may provide a target for future neurorestorative therapies.

REFERENCES

- AHN, M.J., SHERWOOD, E.R., PROUGH, D.S., LIN, C.Y., and DEWITT, D.S. (2004). The effects of traumatic brain injury on cerebral blood flow and brain tissue nitric oxide levels and cytokine expression. *J. Neurotrauma* **21**, 1431–1442.
- BAO, F., and LIU, D. (2002). Peroxynitrite generated in the rat spinal cord induces neuron death and neurological deficits. *Neuroscience* **115**, 839–849.
- BECKMAN, J.S., and CROW, J.P. (1993). Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.* **21**, 330–334.
- BROWN, G.C., and BAL-PRICE, A. (2003). Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol. Neurobiol.* **27**, 325–355.
- CALABRESE, V., BATES, T.E., and STELLA, A.M. (2000). NO synthase and NO-dependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. *Neurochem. Res.* **25**, 1315–1341.
- CAMPOS DE CARVALHO, A., RAMON, F., and SPRAY, C. (1986). Effects of protein reagents on electrotonic coupling in crayfish septate axon. *Am. Physiol. Soc.* C99–C103.
- CHATZIPANTELI, K., GARCIA, R., MARCILLO, A.E., LOOR, K.E., KRAYDIEH, S., and DIETRICH, W.D. (2002). Temporal and segmental distribution of constitutive and inducible nitric oxide synthases after traumatic spinal cord injury: effect of aminoguanidine treatment. *J. Neurotrauma* **19**, 639–651.
- DAVIES, A., HAYES, K.C., and SHI, R. (2006). Recombinant human TNF- α induces concentration-dependent alterations in axonal conduction in mammalian spinal cord. *J. Neurotrauma* **23**, 1261–1273.
- DIAZ-RUIZ, A., VERGARA, P., PEREZ-SEVERIANO, F., et al. (2004). Cyclosporin-A inhibits inducible nitric oxide synthase activity and expression after spinal cord injury in rats. *Neurosci. Lett.* **357**, 49–52.
- DIETZ, V., and COLOMBO, G. (2004). Recovery from spinal cord injury—underlying mechanisms and efficacy of rehabilitation. *Acta Neurochir. Suppl.* **89**, 95–100.
- DITUNNO, J.F., LITTLE, J.W., TESSLER, A., and BURNS, A.S. (2004). Spinal shock revisited: a four-phase model. *Spinal Cord* **42**, 383–395.
- FISHER, C.G., NOONAN, V.K., SMITH, D.E., WING, P.C., DVORAK, M.F., and KWON, B. (2005). Motor recovery, functional status, and health-related quality of life in patients with complete spinal cord injuries. *Spine* **30**, 2200–2207.
- GARTHWAITE, G., GOODWIN, D.A., BATCHELOR, A.M., LEEMING, K., and GARTHWAITE, J. (2002). Nitric oxide toxicity in CNS white matter: an *in vitro* study using rat optic nerve. *Neuroscience* **109**, 145–155.
- GATTI, R.M., RADI, R., and AUGUSTO, O. (1994). Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Lett.* **348**, 287–290.

- HAKIM, T.S., SUGIMORI, K., CAMPORESI, E.M., and ANDERSON, G. (1996). Half-life of nitric oxide in aqueous solutions with and without haemoglobin. *Physiol. Meas.* **17**, 267–277.
- HAYES, K.C., HULL, T.C., DELANEY, G.A., et al. (2002). Elevated serum titers of proinflammatory cytokines and CNS autoantibodies in patients with chronic spinal cord injury. *J. Neurotrauma* **19**, 753–761.
- HAYES, K.C., and KAKULAS, B.A. (1997). Neuropathology of human spinal cord injury sustained in sports-related activities. *J. Neurotrauma* **14**, 235–248.
- HOGG, N., DARLEY-USMAR, V.M., WILSON, M.T., and MONCADA, S. (1992). Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.* **281**, 419–424.
- IADECOLA, C. (1997). Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* **20**, 132–139.
- ISAKSSON, J., FAROOQUE, M., and OLSSON, Y. (2005). Improved functional outcome after spinal cord injury in iNOS-deficient mice. *Spinal Cord* **43**, 167–170.
- KAPOOR, R., DAVIES, M., and SMITH, K.J. (1999). Temporary axonal conduction block and axonal loss in inflammatory neurological disease. A potential role for nitric oxide? *Ann. N.Y. Acad. Sci.* **893**, 304–308.
- KEEFER, L.K., NIMS, R.W., DAVIES, K.M., and WINK, D.A. (1996). “NONOates” (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. *Methods Enzymol.* **268**, 281–293.
- KIRSHBLUM, S., MILLIS, S., MCKINLEY, W., and TULSKY, D. (2004). Late neurologic recovery after traumatic spinal cord injury. *Arch. Phys. Med. Rehabil.* **85**, 1811–1817.
- KÖLLER, H., SIEBLER, M., and HARTUNG, H.P. (1997). Immunologically induced electrophysiological dysfunction: implications for inflammatory diseases of the CNS and PNS. *Prog. Neurobiol.* **52**, 1–26.
- KRONCKE, K.D., FEHSEL, K., and KOLB-BACHOFEN, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection—how, why, when, and where? *Nitric Oxide* **1**, 107–120.
- KWAK, E.K., KIM, J.W., KANG, K.S., et al. (2005). The role of inducible nitric oxide synthase following spinal cord injury in rat. *J. Korean Med. Sci.* **20**, 663–669.
- LANCASTER, J.R., JR. (1996). Diffusion of free nitric oxide. *Methods Enzymol.* **268**, 31–50.
- LAURENT, M., LEPOIVRE, M., and TENU, J.P. (1996). Kinetic modelling of the nitric oxide gradient generated *in vitro* by adherent cells expressing inducible nitric oxide synthase. *Biochem. J.* **314**, 109–113.
- LI, Z., CHAPLEAU, M.W., BATES, J.N., BIELEFELDT, K., LEE, H.C., and ABOUD, F.M. (1998). Nitric oxide as an autocrine regulator of sodium currents in baroreceptor neurons. *Neuron* **20**, 1039–1049.
- LIU, X.Z., XU, X.M., HU, R., et al. (1997). Neuronal and glial apoptosis after traumatic spinal cord injury. *J. Neurosci.* **17**, 5395–5406.
- LU, J., MOOCHHALA, S., SHIRHAN, M., et al. (2003). Nitric oxide induces macrophage apoptosis following traumatic brain injury in rats. *Neurosci. Lett.* **339**, 147–150.
- MARAGOS, C.M., MORLEY, D., WINK, D.A., et al. (1991). Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J. Med. Chem.* **34**, 3242–3247.
- MATSUYAMA, Y., SATO, K., KAMIYA, M., YANO, J., IWATA, H., and ISOBE, K. (1998). Nitric oxide: a possible etiologic factor in spinal cord cavitation. *J. Spinal Disord.* **11**, 248–252.
- MONCADA, S., and ERUSALIMSKY, J.D. (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev. Mol. Cell Biol.* **3**, 214–220.
- NAKAHARA, S., YONE, K., SETOGUCHI, T., et al. (2002). Changes in nitric oxide and expression of nitric oxide synthase in spinal cord after acute traumatic injury in rats. *J. Neurotrauma* **19**, 1467–1474.
- RADI, R., BECKMAN, J.S., BUSH, K.M., and FREEMAN, B.A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481–487.
- REDFORD, E.J., KAPOOR, R., and SMITH, K.J. (1997). Nitric oxide donors reversibly block axonal conduction: demyelinated axons are especially susceptible. *Brain* **120**, 2149–2157.
- REJDAK, K., EIKELNBOOM, M.J., PETZOLD, A., et al. (2004). CSF nitric oxide metabolites are associated with activity and progression of multiple sclerosis. *Neurology* **63**, 1439–1445.
- RENGANATHAN, M., CUMMINS, T.R., and WAXMAN, S.G. (2002). Nitric oxide blocks fast, slow, and persistent Na⁺ channels in C-type DRG neurons by S-nitrosylation. *J. Neurophysiol.* **87**, 761–775.
- ROSE, M.R. (1998). Neurological channelopathies. *BMJ* **316**, 1104–1105.
- SATAKE, K., MATSUYAMA, Y., KAMIYA, M., et al. (2000). Nitric oxide via macrophage iNOS induces apoptosis following traumatic spinal cord injury. *Brain Res. Mol. Brain Res.* **85**, 114–122.
- SATO, T., KAMATA, Y., IRIFUNE, M., and NISHIKAWA, T. (1995). Inhibition of purified (Na⁺,K⁺)-ATPase activity from porcine cerebral cortex by NO generating drugs. *Brain Res.* **704**, 117–120.
- SCHMIDT, K., DESCH, W., KLATT, P., KUKOVETZ, W.R., and MAYER, B. (1997). Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**, 457–462.

- SHI, R., and BLIGHT, A.R. (1996). Compression injury of mammalian spinal cord *in vitro* and the dynamics of action potential conduction failure. *J. Neurophysiol.* **76**, 1572–1580.
- SHI, R., and PRYOR, J.D. (2000). Temperature dependence of membrane sealing following transection in mammalian spinal cord axons. *Neuroscience* **98**, 157–166.
- SHI, R., and PRYOR, J.D. (2002). Pathological changes of isolated spinal cord axons in response to mechanical stretch. *Neuroscience* **110**, 765–777.
- SHRAGER, P. (1977). Slow sodium inactivation in nerve after exposure to sulhydryl blocking reagents. *J. Gen. Physiol.* **69**, 183–202.
- SHRAGER, P., CUSTER, A.W., KAZARINOVA, K., RASBAND, M.N., and MATTSON, D. (1998). Nerve conduction block by nitric oxide that is mediated by the axonal environment. *J. Neurophysiol.* **79**, 529–536.
- SIMMONS, M.L., and MURPHY, S. (1992). Induction of nitric oxide synthase in glial cells. *J. Neurochem.* **59**, 897–905.
- STARKUS, J.G., and SHRAGER, P. (1978). Modification of slow sodium inactivation in nerve after internal perfusion with trypsin. *Am. J. Physiol.* **235**, C238–C244.
- STEINER, L., KRONCKE, K., FEHSEL, K., and KOLB-BACHOFEN, V. (1997). Endothelial cells as cytotoxic effector cells: cytokine-activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide. *Diabetologia* **40**, 150–155.
- STYS, P.K., and LOPACHIN, R.M. (1998). Mechanisms of calcium and sodium fluxes in anoxic myelinated central nervous system axons. *Neuroscience* **82**, 21–32.
- STYS, P.K., WAXMAN, S.G., and RANSOM, B.R. (1992). Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *J. Neurosci.* **12**, 430–439.
- TAKEUCHI, A., ISOBE, K.I., MIYAISHI, O., et al. (1998). Microglial NO induces delayed neuronal death following acute injury in the striatum. *Eur. J. Neurosci.* **10**, 1613–1620.
- TRUDRUNG, P., WIRTH, U., and MENSE, S. (2000). Changes in the number of nitric oxide-synthesizing neurones on both sides of a chronic transection of the rat spinal cord. *Neurosci. Lett.* **287**, 125–128.
- WADA, K., CHATZIPANTELI, K., BUSTO, R., and DIETRICH, W.D. (1998). Role of nitric oxide in traumatic brain injury in the rat. *J. Neurosurg.* **89**, 807–818.
- WANG, X., and FEUERSTEIN, G.Z. (2000). Role of immune and inflammatory mediators in CNS injury. *Drug News Perspect.* **13**, 133–140.
- WAXMAN, S.G. (2001). Acquired channelopathies in nerve injury and MS. *Neurology* **56**, 1621–1627.
- WINK, D.A., DARBYSHIRE, J.F., NIMS, R.W., SAAVEDRA, J.E., and FORD, P.C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O₂ reaction. *Chem. Res. Toxicol.* **6**, 23–27.
- WOOD, J., and GARTHWAITE, J. (1994). Models of the diffusional spread of nitric oxide: implications for neural nitric oxide signalling and its pharmacological properties. *Neuropharmacology* **33**, 1235–1244.
- WU, W., LIUZZI, F.J., SCHINCO, F.P., et al. (1994). Neuronal nitric oxide synthase is induced in spinal neurons by traumatic injury. *Neuroscience* **61**, 719–726.
- YAMADA, K., and NABESHIMA, T. (1997). Simultaneous measurement of nitrite and nitrate levels as indices of nitric oxide release in the cerebellum of conscious rats. *J. Neurochem.* **68**, 1234–1243.
- ZIELASEK, J., TAUSCH, M., TOYKA, K.V., and HARTUNG, H.P. (1992). Production of nitrite by neonatal rat microglial cells/brain macrophages. *Cell Immunol.* **141**, 111–120.

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APPENDIX A

Estimation of nitric oxide concentration using the model described and empirically tested and validated by Schmidt (Schmidt et al., 1997).

In aerobic solutions, the actual concentration of NO depends on the decomposition rate of the donor, the rate of NO formation and the autoxidation rate of NO. In modelling the NO concentration, the following notations are utilized:

t	time (s)
$c_{NO}(t)$	concentration of NO at time t (M)
$c_D(t)$	concentration of the donor at time t (M)
c_0	initial concentration of the donor (M)
e_{NO}	mol of NO released per mol donor
O_2	concentration of oxygen (M)
k_1	rate constant for the decomposition of the donor (s^{-1})
k_2	rate constant for the oxidation of NO ($M^{-2} s^{-1}$)

Provided that the donor (D) is subject to exponential decay, the decomposition reaction will follow first order kinetics with a rate of:

$$d/dt c_D(t) = -k_1 c_D(t) \quad (1)$$

Accordingly, the rate of NO formation is given by

$$d/dt c_{NO}(t) = k_1 c_D(t) e_{NO} \quad (2)$$

In the presence of excess O_2 over NO, autoxidation of NO is governed by a third-order rate law according to Eq. (3)

$$d/dt c_{NO}(t) = -k_2 O_2 c_{NO}(t)^2 \quad (3)$$

The overall reaction can be described by the two differential equations; the previously defined Eq. (1) and

$$d/dt c_{NO}(t) = k_1 c_D(t) e_{NO} - k_2 O_2 c_{NO}(t)^2 \quad (4)$$

with the initial conditions $c_D(0) = c_0$ and $c_{NO}(0) = 0$.

Using a graphical method developed by Schmidt (1997) to obtain a simple estimation of $c_{NO}(t)$, we note that the solution $c_{NO}(t)$ must be recomputed for each given set of parameters. To reduce the number of parameters, new dimensionless variables are used, replacing

$$t \text{ by } \tau = k_1 t \quad (5)$$

$$c_D(t) \text{ by } u_D(\tau) = 1/c_0 c_D(\tau/k_1) \quad (6)$$

$$c_{NO}(t) \text{ by } u_{NO}(\tau) = 1/c_0 e_{NO} c_{NO}(\tau/k_1) \quad (7)$$

With these variables the Eqs. (1) and (4) read

$$d/d\tau u_D(\tau) = -u_D(\tau) \quad (8)$$

$$\begin{aligned} d/d\tau u_{NO}(\tau) &= u_D(\tau) - k_2 O_2 c_0 e_{NO}/k_1 u_{NO}(\tau)^2 \\ &= u_D(\tau) - \alpha u_{NO}(\tau)^2 \end{aligned} \quad (9)$$

with the initial conditions $u_D(0) = 1$ and $u_{NO}(0) = 0$.

The new equations depend only on one parameter α :

$$\alpha = k_2 O_2 c_0 e_{NO}/k_1 \quad (10)$$

The graphical output of Eqs. (8) and (9) is a plot of u_{NO} ($= c_{NO} c_0^{-1} e_{NO}^{-1}$) versus τ ($= k_1 t$) for a given value of α . The family of graphical outputs for the concentrations of Spermine NONOate employed in the present study are shown in Fig. 7A.