British Journal of Pharmacology (1996) 118, 1743-1753

The excitatory and inhibitory modulation of primary afferent fibre-evoked responses of ventral roots in the neonatal rat spinal cord exerted by nitric oxide

[1]Takashi Kurihara & *Koichi Yoshioka

Department of Pharmacology and *Division of Laboratory Science, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113, Japan

1 We investigated the role of nitric oxide (NO) in modulating spinal synaptic responses evoked by electrical and noxious sensory stimuli in the neonatal rat spinal cord *in vitro*.

2 Potentials were recorded extracellularly from a ventral root (L3-L5) of the isolated spinal cord preparation or spinal cord-saphenous nerve-skin preparation of 0- to 2-day-old rats. Spinal reflexes were elicited by electrical stimulation of the ipsilateral dorsal root or by noxious skin stimulation.

3 In the spinal cord preparation, single shock stimulation of a dorsal root at C-fibre strength induced mono-synaptic reflex followed by a slow depolarizing response lasting about 30 s (slow ventral root potential; slow VRP) in the ipsilateral ventral root of the same segment. Bath-application of NO gas-containing medium (10^{-4}-10^{-2} dilution of saturated medium) and NO donors, 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12, 3-300 μM), S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 3-300 μM) and S-nitroso-L-glutathione (GSNO, 3-300 μM), produced an inhibition of the slow VRP and a depolarization of ventral roots. Another NO donor, 3-morpholinosydononimine (SIN-1, 30-300 μM), also depressed the slow VRP but did not depolarize ventral roots. These agents did not affect the mono-synaptic reflex.

4 In the spinal cord-saphenous nerve-skin preparation, application of capsaicin (0.1-0.2 μM) to skin evoked a slow depolarizing response of the L3 ventral root. This slow VRP was depressed by NOC12 (10-300 μM) and SIN-1 (100-300 μM). When the concentration of NOC12 was increased to 1 mM, spontaneous synaptic activities were augmented and the depressant effect of NOC12 on the slow VRP became less pronounced.

5 A NO-scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide(carboxy-PTIO, 100-300 μM) prevented the depressant effect on the dorsal root-evoked slow VRP and ventral root depolarizing effects of NO donors. Carboxy-PTIO increased spontaneous synaptic activities and markedly potentiated the slow VRP. A NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 0.03-1 μM), but not D-NAME (0.03-1 μM), also markedly potentiated the slow VRP and this effect was reversed by L-arginine (300 μM).

6 8-Bromo-cyclic guanosine 3': 5'-monophosphate (8-Br-cyclic GMP, 100-300 μM) produced both an inhibition of the slow VRP and a depolarization of ventral roots. A cyclic GMP-dependent protein kinase inhibitor, KT5823 (0.3 μM), partly inhibited the depressant effects of NO donors and 8-Br-cyclic GMP on the dorsal root-evoked slow VRP. In contrast, KT5823 did not inhibit the depolarizing effects of NO donors.

7 Perfusion of the spinal cord with medium containing tetrodotoxin (0.3 μM) and/or low Ca^{2+} (0.1 mM)-high Mg^{2+} (10 mM) markedly potentiated the depolarizing effect of NO donors. The SNAP-evoked depolarization in the tetrodotoxin-containing low Ca^{2+}-high Mg^{2+} medium was significantly inhibited by excitatory amino acid receptor antagonists D-(-)-2-amino-5-phosphonovaleric acid (30 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM).

8 The present study suggests that inhibitory and excitatory mechanisms meditated by the NO-cyclic GMP cascade are involved in the primary afferent fibre-evoked nociceptive transmission in the neonatal rat spinal cord. The inhibitory mechanism, but not the excitatory mechanism, appears to be partly mediated by cyclic GMP-dependent protein kinase. It is also suggested that Ca^{2+}-independent release of excitatory amino acid neurotransmitters contributes to the depolarizing response to NO of ventral roots.

Keywords: Nitric oxide; nociceptive transmission; cyclic GMP; cyclic GMP-dependent protein kinase; excitatory amino acid release; spinal cord; newborn rat

Introduction

The free radical nitric oxide (NO) is a recently identified highly unorthodox intercellular messenger molecule (Dawson & Snyder, 1994). There is now considerable evidence that NO is implicated in a wide range of physiological functions and pathological phenomena (see Moncada *et al.*, 1991; Moncada & Higgs, 1993; Bredt & Snyder, 1994a, for reviews). NO has been proved to be synthesized from the amino acid L-arginine by a family of enzymes, the NO synthases (NOSs), and is recognized to participate in modulating neuronal functions in the central and peripheral nervous systems (see Schuman & Madison, 1994; Garthwaite & Boulton, 1995, for reviews). In particular, accumulating evidence indicates that NO plays a role in synaptic plasticity (Böhme *et al.*, 1991; O'Dell *et al.*, 1991; Schuman & Madison, 1991; Shibuki & Okada, 1991; see Schuman, 1995, for reviews).

[1]Author for correspondence.

NOS immunocytochemistry and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry have demonstrated the presence of NOS in several specific groups of adult mammalian spinal neurones: superficial and deeper laminae of dorsal horn, area around the central canal (lamina X), intermediolateral cell column and ventral horn (Valtschanoff *et al.*, 1992a,b; Dun *et al.*, 1993; Spike *et al.*, 1993; Zhang *et al.*, 1993; Saito *et al.*, 1994). Developmental studies have shown that the NADPH diaphorase or neuronal NOS staining in the superficial dorsal horn that is observed in the adult rat is almost absent at postnatal day 7 (Kalb & Agostini, 1993; Liuzzi *et al.*, 1993; Soyguder *et al.*, 1994). In contrast, the percentage of neuronal NOS staining in dorsal root ganglion neurones is virtually 100% at embryonic day 12 and diminished gradually to 1% in the adult rat (Bredt & Snyder, 1994b).

Recent papers on rodent behavioral models of pain *in vivo* have shown that administration of NOS inhibitors causes analgesic effects (Moore *et al.*, 1991; Haley *et al.*, 1992; Kitto *et al.*, 1992; Meller *et al.*, 1992a,b; Malmberg & Yaksh, 1993) and suggested that NO probably released in laminae II and III of the dorsal horn may contribute to the development of hyperalgesia and allodynia (see McMahon *et al.*, 1993; Meller & Gebhart, 1993, for reviews). However, several studies have demonstrated analgesic effects of L-arginine but not D-arginine (Takagi *et al.*, 1990; Moore *et al.*, 1991; Haley *et al.*, 1992; Zhuo *et al.*, 1993) and analgesic roles of NO (Zhuo *et al.*, 1993; Iwamoto & Marion, 1994; Kreeger *et al.*, 1994; Lothe *et al.*, 1994) have also been suggested.

In an attempt to resolve these apparently conflicting notions on the role of NO in spinal nociceptive transmission, we have carried out experiments in the isolated spinal cord preparation of the neonatal rat (Akagi *et al.*, 1985; Yanagisawa *et al.*, 1992). This preparation enables us to obtain stable extracellular recording from ventral roots and thus to carry out detailed pharmacological analyses of both responses to nerve stimulation and responses to exogenously applied agents. Activation of primary afferent fibres by either electrical stimulation at C-fibre strength or peripheral noxious stimulation evokes a depolarization of a slow time course in ventral roots and this depolarization is markedly depressed by tachykinin antagonists as well as opioid agonists (Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989; Yanagisawa *et al.*, 1992; Guo *et al.*, 1993; Hosoki *et al.*, 1994). These and other lines of evidence suggest that this depolarization, hereafter referred to as the slow ventral root potential (VRP), represents a C-fibre-evoked nociceptive response in which tachykininergic primary afferents are involved (Otsuka & Yanagisawa, 1987). Using this preparation in the present study we examined the effects of NO-related agents on the spinal synaptic responses evoked by electrical and noxious sensory stimuli. We also carried out pharmacological experiments to investigate the synaptic and intracellular mechanisms of the NO-evoked responses. Preliminary accounts of this study were presented at the 67th annual meeting of the Japanese Pharmacological Society and the 18th annual meeting of the Japan Neuroscience Society (Kurihara & Yoshioka, 1994a,b).

Methods

Preparations and electrophysiology

Isolated spinal cord preparation The isolated spinal cord preparation of neonatal Wistar rats of either sex (0–2-day-old) was used as described previously (Akagi *et al.*, 1985). Under ether anaesthesia, the spinal cord below the middle thoracic level together with spinal nerve roots (L3–L5) was isolated, hemisected, and placed in a recording chamber of 0.3 ml volume. The chamber was perfused (2.5 ml min^{-1}) with artificial cerebrospinal fluid (CSF) saturated with a gas mixture of 95% O_2: 5% CO_2 at room temperature (25–27°C). The composition of artificial CSF was as follows (mM): NaCl 138.6, KCl 3.35, $NaHCO_3$ 20.9, glucose 10.0, $CaCl_2$ 1.25, $MgCl_2$ 1.15.

A tight-fitting suction electrode was used for extracellular recording from a ventral root (L3–L5). Another suction electrode was used for electrical stimulation of the dorsal root of the same segment (single shocks with square pulses of 500 μs in duration and 15–25 V in amplitude, supramaximum for mono-synaptic reflex and slow VRP; positive voltage was applied to the inside of the electrode). Potential changes of the ventral root were led to a d.c. amplifier and then to a pen recorder and a computer recording device (Axotape version 2, Axon Instruments). The magnitude of the depolarizing response to the electrical stimulation and depolarizing agents was expressed as the integrated area (mV min) of the depolarization. The magnitude of the control slow VRP was 0.34 ± 0.02 mV min ($n = 34$; mean ± s.e.mean). The spinal reflexes of fast time courses were stored in a transient memory device and then recorded on the pen recorder with an expanded time-scale.

Isolated spinal cord-saphenous nerve-skin preparation The hemisected spinal cord below the middle thoracic level was isolated from 0–2-day-old rats together with the attached L3–L5 ventral roots and dorsal roots, the latter remaining connected with the dorsal root ganglia and the femoral and saphenous nerves and a piece of skin (approximately 5 × 5 mm) of the hind limb (Yanagisawa *et al.*, 1992). The recording chamber was made from Sylgard and consisted of two wells, which were independently perfused (2.5 ml min^{-1}) with artificial CSF at room temperature (25–27°C). The concentration of $MgCl_2$ in artificial CSF was increased to 2 mM in order to depress spontaneous activity. The spinal cord was placed in one well (0.3 ml volume) and the skin was placed with an inside surface upwards in the neighboring well (0.1 ml volume). The saphenous nerve was led through a slit (2 mm width) in a thin septum (2 mm width) into the skin well. The break in the septum was sealed with Vaseline. Capsaicin (0.1–0.2 μM) was applied to the skin for 30 s by perfusing the skin well with the medium containing the drug at intervals of 40–45 min to minimize tachyphylaxis. The evoked responses were recorded from the L3 ventral root on the pen recorder and the computer recording device. The magnitude of each depolarizing response was estimated as the integrated area as described above. The magnitude of the control slow VRP evoked by capsaicin was 0.81 ± 0.07 mV min ($n = 12$).

Drugs

The following drugs were used: N-acetyl-D,L-penicillamine (NAP) was purchased from Aldrich Chemical Company, Inc., Tokyo, Japan; D-(−)-2-amino-5-phosphonovaleric acid (D-APV) was from Cambridge Research Biochemicals Ltd., Cheshire, UK; L-arginine was from Takara Kohsan Co. Ltd., Tokyo, Japan; 8-bromo adenosine 3′: 5′-cyclic monophosphate (8-Br-cyclic AMP), 8-bromo guanosine 3′:5′-cyclic monophosphate (8-Br cyclic GMP), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-D-arginine methyl ester (D-NAME), sodium nitrate and sodium nitrite were from Sigma Chemical Company, St. Louis, MO, USA; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1 - oxyl-3 - oxide (carboxy-PTIO), 1-hydroxy-2-oxo-3- (N-ethyl-2-aminoethyl) -3-ethyl-1-triazene (NOC12), 3-morpholino-sydononimine (SIN-1) and S-nitroso-N-acetyl-D, L-penicillamine (SNAP) were from Dojindo Laboratories, Kumamoto, Japan; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from Research Biochemicals Inc., Natick, MA, U.S.A.; KT5823 (8R*, 95*, 115*)-9-methoxy-9-methoxycarbonyl-2,8-dimethyl-3,9,10-tetrahydro - 8,11-epoxy-1H,8H,11H - 2,7b,11a - triazadibenzo [a,g]cycloocta[c,d,e] trinden-1-one) was from Kyowa Medex Co., Ltd., Tokyo, Japan; S-nitroso-L-glutathione (GSNO) was from Tocris Cookson Ltd, Bristol, UK; tetrodotoxin (TTX) was from Sankyo Co., Ltd., Tokyo, Japan.

NO gas with 99% purity was obtained from Suzusho

Medical, Tokyo, Japan. To prepare NO-saturated solution. NO gas was bubbled, for 30–40 min, into artificial CSF, which had been deaerated by bubbling with 95% N_2: 5% CO_2 gas for 2–3 h. Appropriate dilutions were made immediately before each experiment.

Stock solutions (100 mM or 1 M) of carboxy-PTIO, GSNO, NAP, NOC12, SIN-1 and SNAP were prepared fresh each day, diluted to desired concentrations in artificial CSF immediately before each experiment, and used within 2 h. To make stock solutions, carboxy-PTIO were dissolved in pH 9 artificial CSF (adjusted with 2 M NaOH), NAP, SNAP and KT5823 in dimethyl sulphoxide (DMSO), NOC12 in 100 mM NaOH and all other drugs in artificial CSF. Stock solutions of carboxy-PTIO, GSNO and SNAP were kept from light throughout the experiment.

Drug application protocol

All drugs were applied to the spinal cord or the skin by perfusion. The inhibitory effects of NO gas-containing medium, NO donors and cyclic nucleotides on the dorsal root-evoked slow VRP were evaluated by comparing the averaged magnitude of three control slow VRPs and the two responses under the steady effects of these agents. The effects on the capsaicin-evoked slow VRP were evaluated by comparing two control slow VRPs and the one response. In order to obtain steady depressant effects the compounds were applied to the spinal cord for 5 min except for SIN-1 and cyclic nucleotides, which were applied for 8 min because of the slow onset of the effects. In the experiments to examine depolarizing effects on ventral roots of NO gas-containing medium, NO donors and cyclic nucleotides, these compounds were applied to the spinal cord for 30 s or 1 min at intervals of 40–50 min to minimize tachyphylaxis.

Statistical analysis

Experimental data are expressed as mean values ± s.e.mean and tested according to Student's t test. A P value less than 0.05 was considered significant.

Results

Effects of NO gas-containing medium and NO donors in the neonatal rat spinal cord

We first examined the effects of NO gas-containing medium and several structurally different NO donors on electrical activities of ventral roots in the isolated spinal cord preparation of neonatal rats. Electrical stimulation of a dorsal root evoked mono-synaptic reflex followed by a prolonged depolarization lasting about 30 s (slow VRP) on the ipsilateral ventral root of the same segment. Bath application of NO gas-containing medium (10^{-4}–10^{-2} dilution of saturated medium; Figure 1a), NOC12 (3–300 μM; Figure 1b), GSNO (3–300 μM; data not shown) and SNAP (3–300 μM; data not shown) to the spinal cord evoked two distinct effects. One effect was an inhibition of the slow VRP and the other was a depolarization of ventral roots. NO gas-containing medium and NO donors also suppressed spontaneous activities, but did not affect the mono-synaptic reflex (Figure 1a and b). The inhibitory effects of NO gas and NO donors reached a maximum within 2–3 min and almost completely recovered after 10–15 min washouts. NO gas, GSNO, NOC12 and SNAP depressed the slow VRP to 45.8 ± 4.80 ($P<0.01$), 49.0 ± 12.1 ($P<0.01$), 50.2 ± 2.83 ($P<0.01$) and 51.0 ± 6.77% ($P<0.01$) of the control response at the highest concentrations, respectively (Figure 2a and b; $n=4$–5). Comparing the

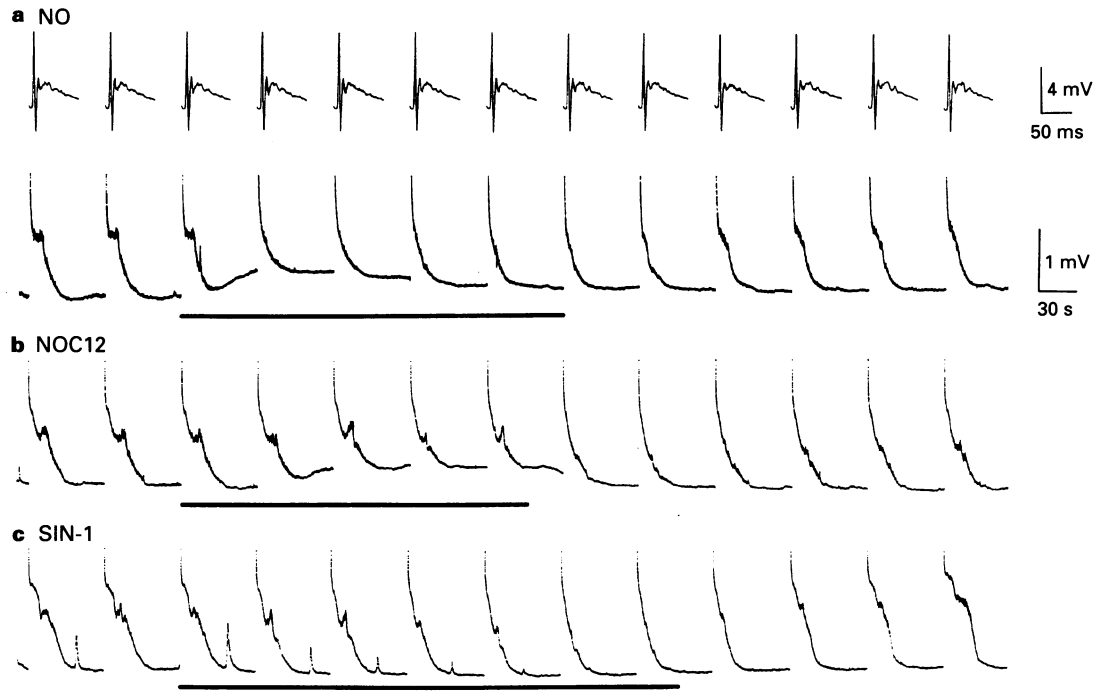

Figure 1 Effects of NO-gas containing medium and NO donors in the neonatal rat spinal cord. NO gas-containing medium (10^{-2} dilution, a), 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene(NOC12; 100 μM, b) and 3-morpholinosydononimine (SIN-1; 100 μM, c) were applied during the periods indicated by the bars. NO gas and NOC12 evoked two distinct responses; an inhibition of the slow ventral root potential (VRP) and a depolarization of ventral roots. The mono-synaptic reflex (the upper trace of a) was, however, little affected. In contrast, SIN-1 inhibited the slow VRP without depolarization. Single-shock stimuli of supramaximal intensity were applied to the L4 dorsal root every 1 min and the resulting reflexes were recorded from the ipsilateral ventral root of the same segment. The upper trace of (a) shows the records of the fast reflex responses during 52 ms post-stimulus periods, which were stored in a memory device in a.c. mode and then recorded on a pen-recorder on a 1000 times expanded time-base. Initial sharp spikes represent the mono-synaptic reflexes. Other traces show continuous chart records of the d.c. potential. Records in (a), (b) and (c) were taken from different preparations.

concentration-response curve of NO gas in Figure 2a with those of NO donors in Figure 2b, we may conclude that the inhibitory effects of these NO donors at 10 μM were roughly equivalent to that of NO-gas medium at 10^{-4} dilution. Thus, assuming that the saturated NO-gas medium contain about 2 mM NO (Feelisch, 1991), 10^{-4} diluted NO solution and solutions containing 10 μM of these NO donors correspond to maximally 0.2 μM NO solution.

Among the NO donors examined in this study, SIN-1 (30–300 μM) was unique in that it evoked no ventral root depolarization, although it inhibited the slow VRP (to 50.6±3.25% of the control response; $n=5$; $P<0.01$) and spontaneous activities (Figure 1c). The onset of the inhibitory action of SIN-1 was slow and it took more than 5 min to reach the maximum effect (Figures 1c and 2c). Like other NO donors, SIN-1 did not affect the mono-synaptic reflex (data not shown). NAP (300 μM), which does not have the S-nitroso group (-S-NO) contained in SNAP, had no effect on the slow VRP (112.7±15.7% of the control response; $n=3$) and did not evoke ventral root depolarization (data not shown). Similarly, the maximum final concentrations of vehicles used to dilute drugs (0.1% DMSO and 100 μM NaOH) had no effect on the spinal reflexes.

Higher concentrations of NO donors were necessary to evoke the depolarization of ventral roots than to evoke the inhibition of slow VRP. Thus, 3 μM of GSNO and NOC12 was sufficient to evoke a significant ($P<0.05$) inhibition of the slow VRP (Figure 2b), whereas more than 10 μM was necessary to depolarize ventral roots (Figure 2d). Furthermore, successive applications of the NO donors caused a decline of the depolarizing effects and more than 40 min intervals between the applications were necessary to minimize the tachyphylaxis. In contrast, the inhibitory effects on the slow VRP were quite stable and upon prolonged application of the NO donors for up to 45 min there were no declines in the inhibition (data not shown).

Effects of NO donors on the slow VRP evoked by noxious skin stimulation

In the spinal cord-saphenous nerve-skin preparation, application of capsaicin (0.1–0.2 μM for 30 s) to skin produced a depolarizing response of the L3 ventral root of a slow time course (Figure 3). This response was markedly depressed by perfusion of SIN-1 (100–300 μM; Figure 3a) and NOC12 (0.01–1 mM; Figure 3b) to the spinal cord. SIN-1 (300 μM; $n=4$) and NOC12 (100 μM; $n=7$) depressed the capsaicin-evoked slow VRP to 12.1±2.42% and 22.9±8.17% of the control response, respectively. However, at a higher concentration (1 mM) of NOC12, the depressant effect became smaller in 5 out of 7 preparations (41.8±7.28% of the control response; $P<0.05$ compared with the value at 100 μM; Figure 3b).

Effects of a NOS inhibitor on the dorsal root-evoked slow VRP

To examine whether endogenous NO is involved in modulating the dorsal root-evoked slow VRP, we tested the effect of a NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME).

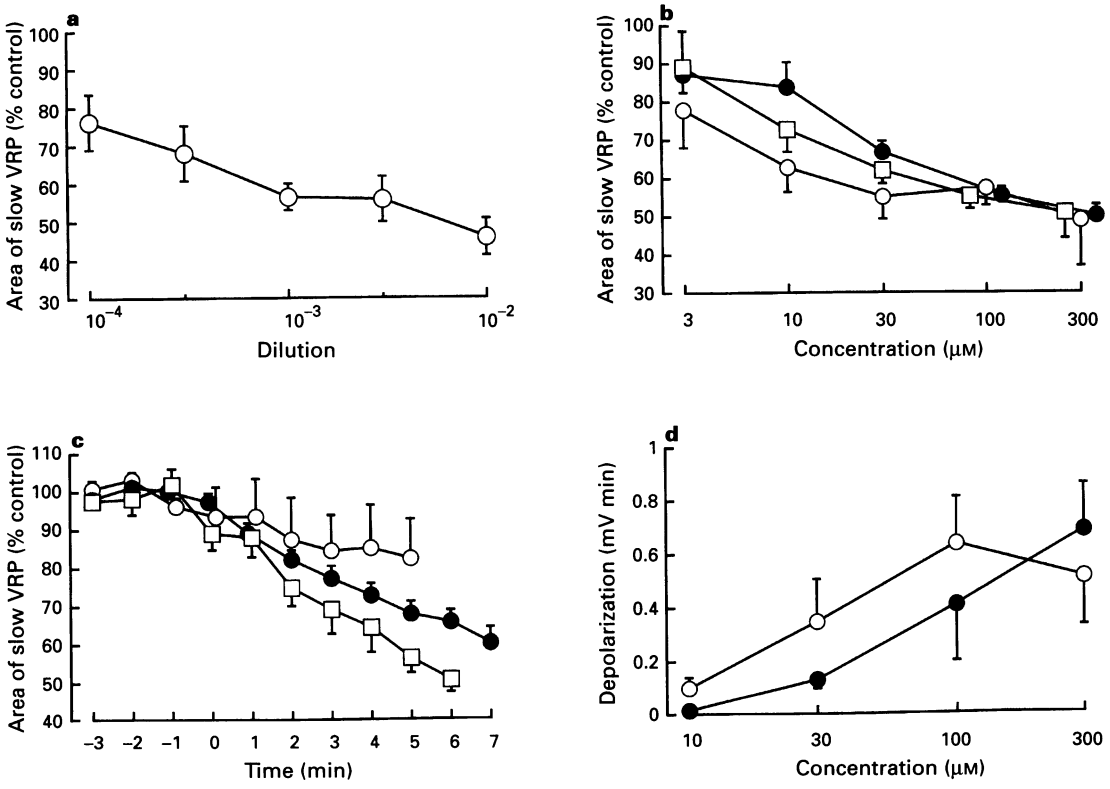

Figure 2 Inhibition of slow ventral root potential (VRP) by NO gas-containing medium and NO donors and depolarization of ventral roots by NO donors. (a) Concentration-inhibition curve for NO gas-containing medium. The abscissa represents the magnification of dilution. (b) Concentration-inhibition curves for S-nitroso-L-glutathione (GSNO; ○), NOC12 (●) and S-nitroso-N-acetyl-D, L-penicillamine (SNAP; □). (c) Time course of inhibition of slow VRP by SIN-1 (○, 30 μM; ●, 100 μM; and □, 300 μM). Application of SIN-1 was started at time 0. (d) Concentration-depolarization curves for SNAP (○) and NOC12 (●). In (d) NO donors were applied to the spinal cord for 1 min. In (a), (b) and (c), the ordinate scale represents the magnitude of dorsal root-evoked slow VRP, which was measured as the integrated area of depolarization and expressed as percentage of the averaged magnitude of the three control responses. In (d) the ordinate scale represents the area of depolarization in mVmin. Each point and vertical bar represent the mean and s.e.mean ($n=3-16$). The positions of some symbols are horizontally adjusted to avoid overlapping. For key to abbreviations used see Figure 1 legend.

L-NAME at 1 μM markedly potentiated the slow VRP to 167.2±10.8% of the control response ($n=4$) whereas L-arginine (100 μM) significantly reversed the potentiating effect of L-NAME ($n=4$; Figure 4). D-NAME (0.03–1 μM), in contrast, did not affect on the slow VRP ($n=4$; Figure 4).

Effects of a NO scavenger on the dorsal root-evoked slow VRP and the responses to NO donors

We further examined the effect of a recently introduced NO scavenger, carboxy-PTIO (Akaike *et al.*, 1993), on the dorsal root-evoked slow VRP. Carboxy-PTIO is an organic radical and it converts NO into nitrogen dioxide ($NO_2^{\bullet}$) via radical-radical reaction. As shown in Figure 5, carboxy-PTIO (100–300 μM) increased the frequency of spontaneous activities and markedly potentiated the slow VRP to 175±18.8% of the control response at 200 μM ($n=6$). Concentrations of carboxy-PTIO exceeding 200 μM evoked too frequent spontaneous activities to estimate accurately the magnitude of the slow VRP (Figure 5c).

Carboxy-PTIO (100–200 μM) significantly reduced the inhibition of the slow VRP induced by SNAP (100 μM; from 53.9±3.33% to 79.6±5.81% of the control response; $n=4$) and NOC12 (100 μM; from 59.4±4.01% to 80.2±2.93%; $n=4$), and almost completely blocked the action of SIN-1 (100 μM) (Figure 6). Carboxy-PTIO (300 μM) also markedly blocked the ventral root depolarization evoked by SNAP (100 μM) to 8.29±3.93% of the control response ($n=3$; Figure 7) and that by NOC12 (100 μM) to 9.58±4.65% ($n=3$) in the presence of TTX (0.3 μM). These observations suggest that NO is involved in the inhibition of slow VRP and ventral root depolarization evoked by NO donors. In accord with this notion, neither sodium nitrite (300 μM) nor sodium nitrate (300 μM) evoked inhibition of slow VRP and depolarization of ventral roots (data not shown).

Effects of 8-Br-cyclic GMP and a cyclic GMP dependent protein kinase inhibitor

The primary cellular response to NO seems to be activation of the soluble isoforms of guanylyl cyclase (sGC) and subsequent production of cyclic GMP (see Schmidt *et al.*, 1993; Vincent, 1994; for reviews). We therefore examined the effect of a membrane permeable analogue of cyclic GMP, 8-Br-cyclic GMP. Like NO gas-containing medium and NO donors, 8-Br-cyclic GMP (100–300 μM) evoked both an inhibition of the dorsal root-evoked slow VRP and a depolarization of ventral roots without affecting mono-synaptic reflex (Figure 8). At 300 μM, 8-Br-cyclic GMP markedly depressed the slow VRP to 29.8±3.84% of the control response ($n=5$). These results suggest that both inhibitory and excitatory effects of NO gas and NO donors in the neonatal rat spinal cord are mediated by the sGC-cyclic GMP pathway. 8-Br-cyclic AMP (300–500 μM), an analogue of cyclic AMP, evoked a depolarization of the ventral roots with a similar potency to 8-Br-cyclic GMP but induced only slight inhibition of the slow VRP (82.8±6.07% of the control response at 500 μM; $n=4$, $P<0.01$).

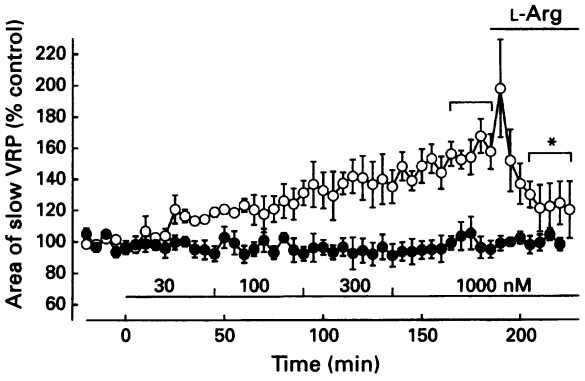

Figure 4 Effect of a NO synthase inhibitor, L-NAME, on the slow ventral root potential (VRP). Four increasing concentrations of L-NAME (○) and D-NAME (●) were cumulatively applied to the spinal cord. In the presence of L-NAME (1 μM), L-arginine (L-Arg; 100 μM) was applied during the period indicated by the bar. L-Arginine reversed the potentiating effect of L-NAME. Ordinate scale: the magnitude of dorsal root-evoked slow VRP was measured as the integrated area of depolarization and expressed as percentage of the averaged magnitude of the three control responses. Each point and vertical bar represent the mean and s.e.mean ($n=4$). *Statistically significant difference between the average values of the points within the brackets ($P<0.05$).

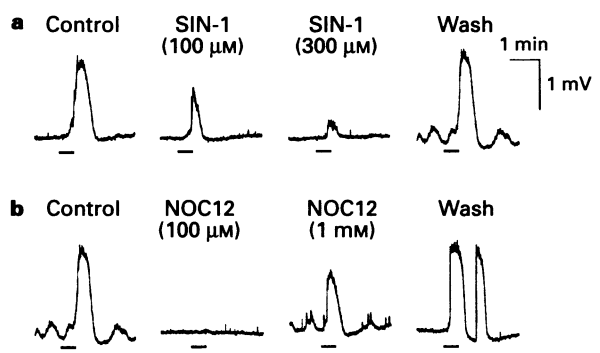

Figure 3 Effects of NO donors on the slow ventral root potential (VRP) evoked by noxious skin stimulation in an isolated spinal cord-saphenous nerve-skin preparation. Capsaicin (0.1 μM) was applied to the skin during the periods (30 s) indicated by the bars. The records in the middle two columns were taken after pretreatment of the spinal cord with SIN-1 (a) or NOC12 (b) for 5 min. Records in (a) and (b) were taken from the same preparation. For key to abbreviations used see Figure 1 legend.

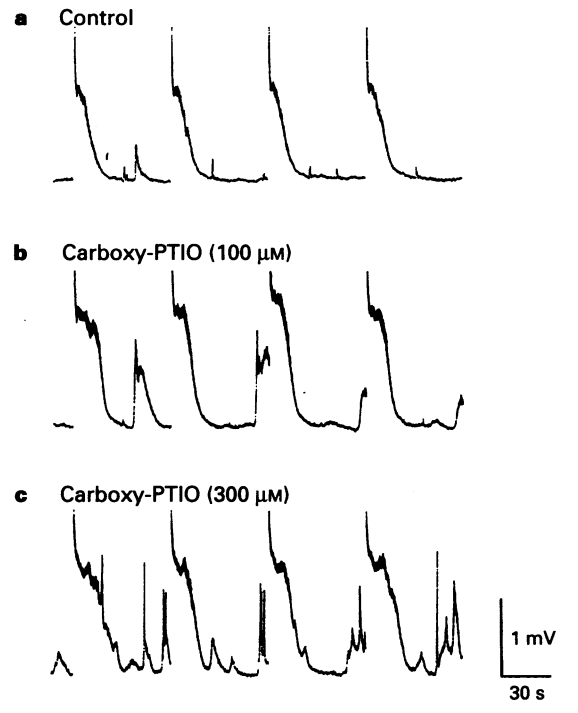

Figure 5 Effect of a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO), on the slow ventral root potential (VRP). (a) In normal artificial CSF. (b) and (c) In the presence of carboxy-PTIO (100 μM and 300 μM, respectively).

To investigate the possible involvement of cyclic GMP-dependent protein kinase (PKG) in the actions of NO donors, the effects of an inhibitor of PKG, KT5823 (Nakanishi, 1989), were examined. The inhibition constant values (K_i in μM) for KT5823 have been estimated as 0.234 for PKG, 4.0 for protein kinase C, and larger than 10.0 for cyclic AMP-dependent protein kinase and myosin light chain kinase (Nakanishi, 1989). Application of KT5823 (0.3 μM) to the spinal cord slightly but significantly depressed the dorsal root-evoked slow VRP (to 84.1±5.51% of the control response; $n=11$, $P<0.01$). KT5823 (0.3 μM) almost completely blocked the inhibition of slow VRP evoked by SIN-1 (100 μM; Figure 9a) in a reversible manner and partially blocked those of NOC12 (100 μM; from 50.5±1.98% of the control response to 75.1±5.91% in the presence of KT5823; $n=5$, $P<0.01$) and GSNO (100 μM; $n=2$; data not shown). KT5823 also partly blocked the inhibitory effect of 8-Br-cyclic GMP (100 μM;

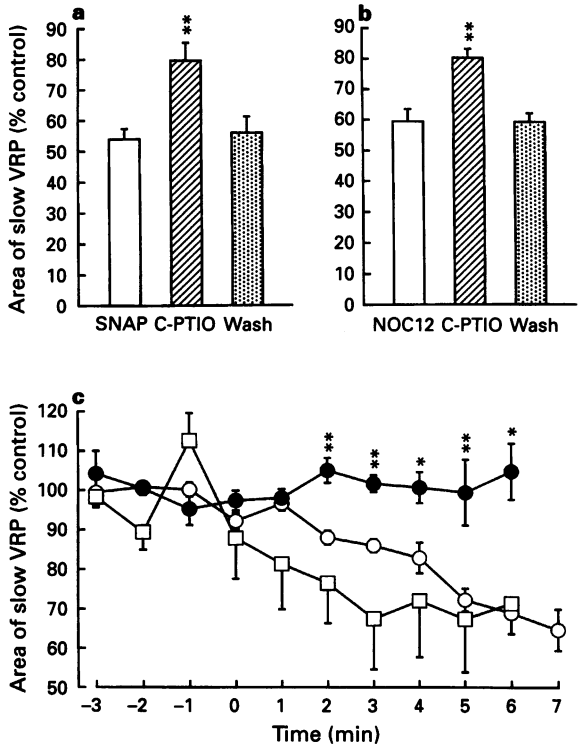

Figure 6 Antagonism by carboxy-PTIO (C-PTIO) of the slow ventral root potential (VRP) inhibition evoked by SNAP (100 μM, a), NOC12 (100 μM, b) and SIN-1 (100 μM, c). In (a) and (b), open column represents the inhibitory effect of NO donors in normal medium; hatched column, in the presence of C-PTIO (200 μM); stippled column, after washing out C-PTIO. In (c) the abscissa scale represents the time course, in which the application of SIN-1 was started at time 0. (○) The inhibitory effect of SIN-1 in normal medium; (●) in the presence of C-PTIO (200 μM); (□) about 30 min after washing out C-PTIO. Ordinate scale: the magnitude of dorsal root-evoked slow VRP, which was measured as the integrated area of depolarization and expressed as percentage of the averaged magnitude of the three control responses. Each point and vertical bar represent the mean and s.e.mean ($n=3-4$). $^*P<0.05$, $^{**}P<0.01$, when compared with the control value. For key to abbreviations used see legends of Figures 1 and 5.

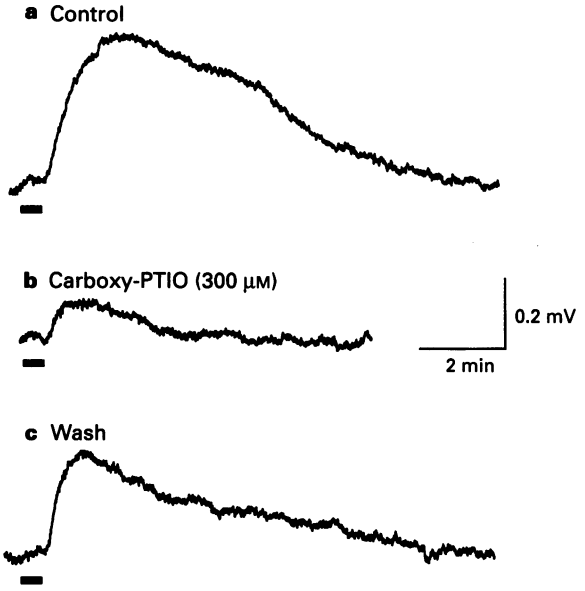

Figure 7 Antagonism by carboxy-PTIO of the ventral root depolarization evoked by SNAP. Carboxy-PTIO (300 μM) largely blocked the SNAP-evoked depolarization. (a) Control response. (b) Response in the presence of carboxy-PTIO (300 μM); (c) 30 min after washing out carboxy-PTIO. SNAP (100 μM) was bath-applied to the spinal cord during the period (30 s) indicated by the bars. The spinal cord was perfused with artificial CSF containing tetrodotoxin (0.3 μM) throughout the experiment. For key to abbreviations used see legend of Figures 2 and 5.

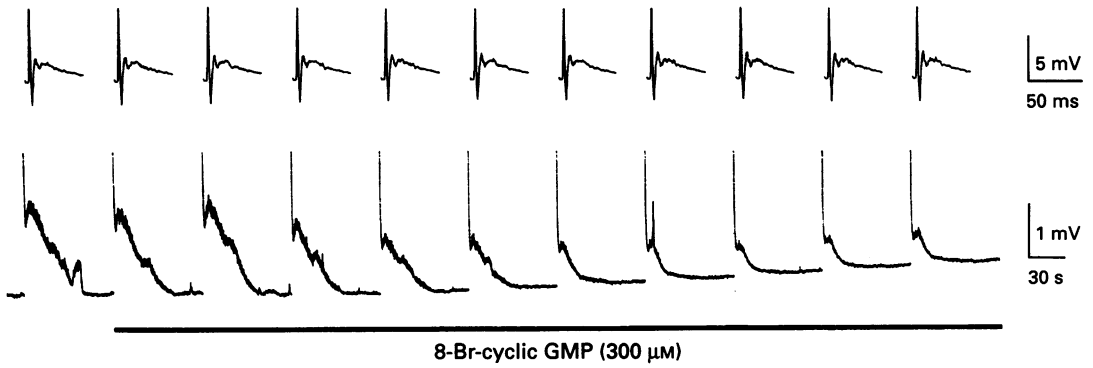

Figure 8 Effects of 8-Br-cyclic GMP. Typical sample record. 8-Br-cyclic GMP was applied during the period (10 min) indicated by the bar. Note that 8-Br-cyclic GMP (300 μM) inhibited the slow ventral root potential (VRP) and caused a slight depolarization of the ventral root. In contrast, 8-Br-cyclic GMP had little effect on the mono-synaptic reflex (the upper trace). The conditions of stimulation and recording were the same as in Figure 1.

$n=3$; Figure 9b). In contrast, KT5823 (0.3 μM) did not change the magnitudes of the depolarizing responses evoked by NOC12 (100 μM; $n=4$) and GSNO (100 μM; $n=3$) (111.7±8.71% and 99.6±4.57% of the control response, respectively) in normal artificial CSF.

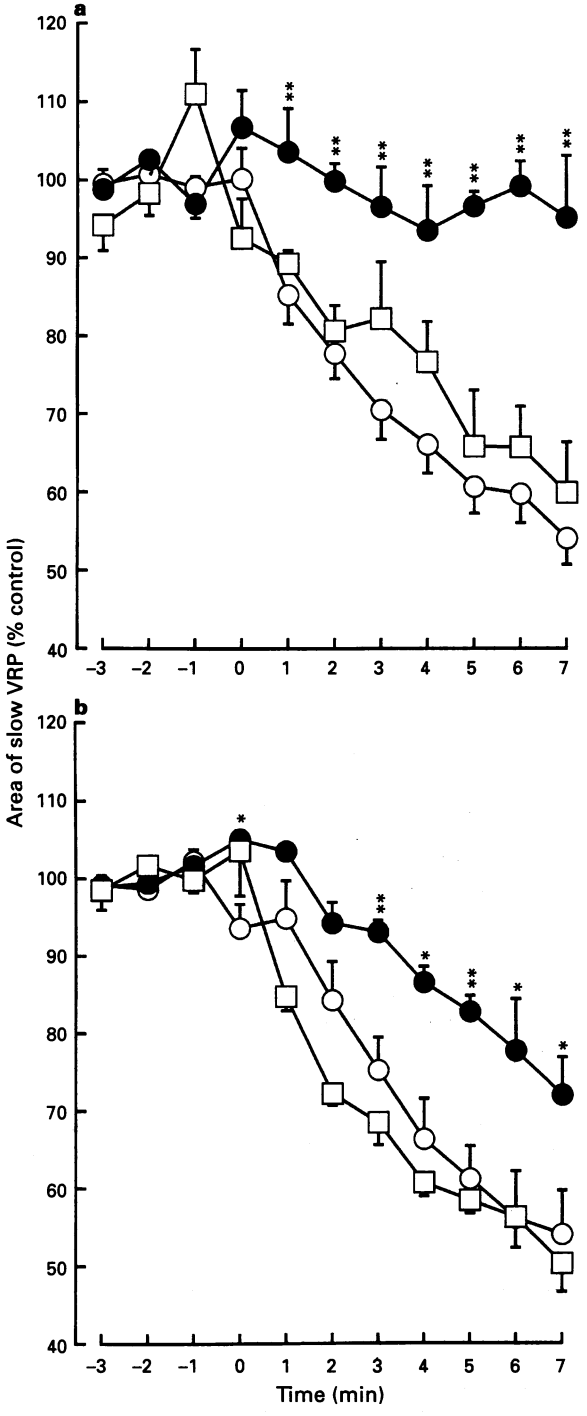

Figure 9 Antagonism by KT5823 of the inhibition of slow ventral root potential (VRP) induced by 3-morpholinosydononimine (SIN-1) (a) and 8-Br-cyclic GMP (b). (○) Control response induced by SIN-1 (100 μM, a) and 8-Br-cyclic GMP (100 μM, b); (●) about 120 min after adding KT5823 (0.3 μM); (□) about 80 min after washing out KT5823. Ordinate scales: the magnitude of dorsal root-evoked slow VRP was measured as the integrated area of depolarization and expressed as percentage of the averaged magnitude of the three control responses. Each point and vertical bar represent the mean and s.e.mean ($n=3-5$). $^*P<0.05$, $^{**}P<0.01$, when compared with the control value.

Depolarizing action of NO donors in the presence of tetrodotoxin and/or low Ca^{2+}-high Mg^{2+} medium

The ventral root depolarization evoked by NO gas-containing medium and NO donors in normal artificial CSF presumably reflects the depolarization of motoneurone cell bodies and results from a summation of the trans-synaptic action of the compounds through spinal interneurones and direct action on motoneurones (Otsuka & Yanagisawa, 1988). In an attempt to estimate the relative contributions of the direct action and trans-synaptic action, we examined the effects of GSNO, NOC12 and SNAP in the presence of tetrodotoxin (TTX; 0.3 μM). The TTX-containing medium, which completely blocked the spinal reflexes evoked by dorsal root stimulation, did not reduce but rather significantly potentiated the depolarizing effects of SNAP ($P<0.05$; Figure 10), GSNO (data not shown) and NOC12 (data not shown). We also examined the depolarizing effects of the NO donors in low Ca^{2+} (0.1 mM)-high Mg^{2+} (10 mM) medium or the low Ca^{2+}-high Mg^{2+} medium containing TTX (0.3 μM), and obtained essentially the same results (data not shown).

Although the interpretation of the above observation is not simple, the clear effect of the block of synaptic transmission on the NO mediated depolarization suggests involvement of neurotransmitter release. We therefore examined effects of several receptor antagonists. Among them, we found that excitatory amino acid (EAA) receptor antagonists, D-APV (30 μM) and CNQX (10 μM), significantly reduced the depolarization of ventral roots evoked by SNAP (300 μM) in the low Ca^{2+}-high Mg^{2+} medium containing TTX (Figure 11). D-APV alone reduced the depolarization to 64.8±7.51% of the control response ($n=4$, $P<0.01$) and the mixture of D-APV and CNQX further reduced it to 31.0±12.7% ($n=4$, $P<0.01$).

Discussion

In this study we found that NO gas-containing medium and various NO donors evoked two responses, i.e. inhibition of slow VRPs and depolarization of ventral roots, in the neonatal rat spinal cord. Several differences were observed in the properties of the two responses. First, compared with the inhibitory response, higher concentrations of NO donors (more than 10 μM) were necessary to evoke the depolarizing response. Secondly, tachyphylaxis was observed in the depolarizing, but not inhibitory, response. Finally, the PKG inhibitor KT5823 suppressed the inhibitory, but not depolarizing, response. These results suggest that these two responses are distinct, involving different intracellular mechanisms.

Both the NOS inhibitor L-NAME and the NO scavenger carboxy-PTIO markedly augmented the dorsal root-evoked slow VRP. This result suggests that NO may be produced endogenously and exert inhibitory influence on these spinal nociceptive responses. However, at the highest concentration (1 mM) of NOC12 employed in this study, the depressant effect on the capsaicin evoked-slow VRP became smaller than at 100 μM (see Figure 3b). From these results we suppose that NO predominantly inhibits this spinal nociceptive pathway at relatively low concentrations (submicromolar range at a rough estimate; see Results), but NO also exerts a facilitatory influence at higher concentrations. This concentration-dependent, inhibitory and excitatory modulation may explain the apparently conflicting findings of previous studies on the role of NO in spinal nociception (see Introduction).

Possible postnatal changes in NO-mediated responses, however, must also be considered. In this study we used spinal cord preparations of 0–2-day-old rats. In contrast to the augmentation of the slow VRP by L-NAME observed in this study, suppression of nociceptive responses by NOS inhibitors were obtained in previous *in vivo* experiments with adult animals (see Introduction). One explanation for this apparent discrepancy may be that excitatory effect of NO is predominant in adult animals. In support of this notion, in our

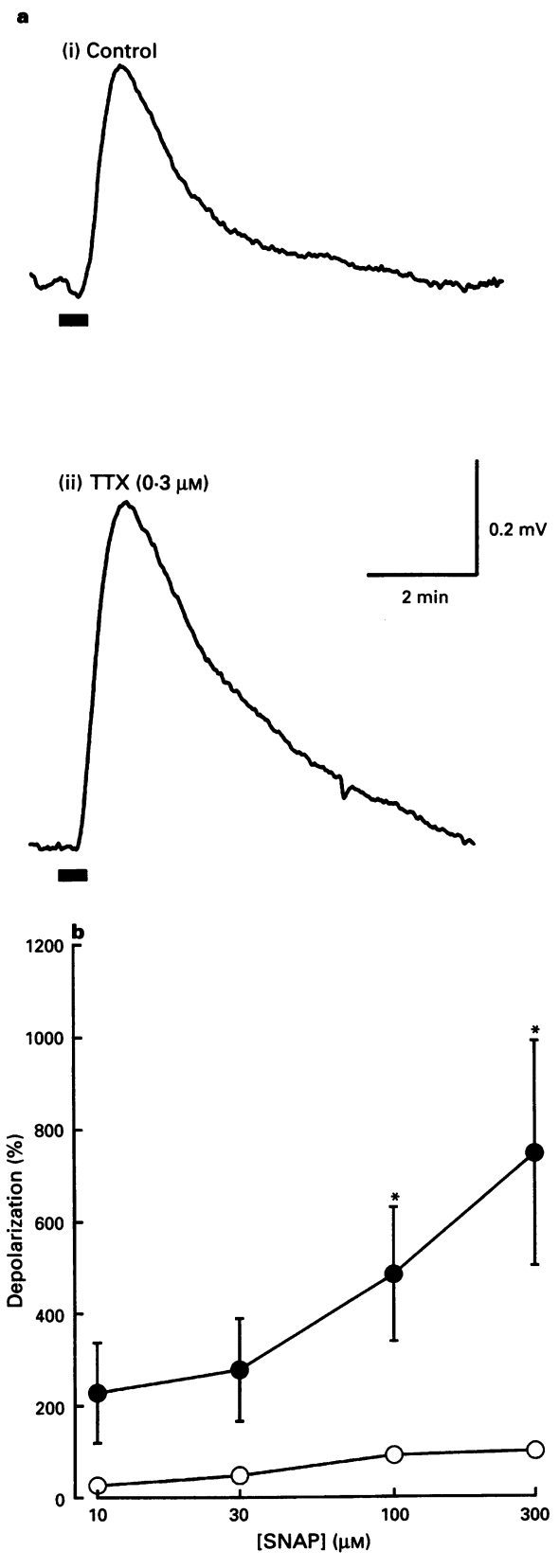

Figure 10 Effect of tetrodotoxin (TTX) on the depolarization of ventral roots evoked by SNAP. (a) Typical sample record in normal medium (i) and in the presence of TTX (0.3 μM) (ii). SNAP (100 μM) was bath applied to the spinal cord during the period (30 s) indicated by the bars. (b) Concentration-response curves: (○) control response in normal medium; (●) in the presence of TTX. Note that the depolarizing effect of SNAP was more pronounced in the presence of TTX. Each point and vertical bar represent the mean and s.e.mean ($n=5-7$); vertical bars are indicated only when larger than symbols. *$P<0.05$, when compared with the control value. For key to abbreviations used see legend of Figure 2.

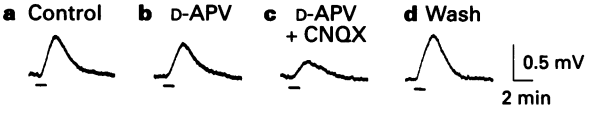

Figure 11 Effects of excitatory amino acid receptor antagonists on the ventral root depolarization evoked by SNAP in low Ca^{2+}-high Mg^{2+} medium containing tetrodotoxin (TTX). (a) Control response. (b) Response in the presence of D-(−)-2-amino-5-phosphonovaleric acid (D-APV; 30 μM). (c) Response in the presence of a mixture of D-APV (30 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM). (d) 70 min after washing out D-APV and CNQX. S-nitroso-N-acetyl-D, L-penicillamine (SNAP; 300 μM) was bath applied to the spinal cord during the period (1 min) indicated by the bars. The spinal cord was perfused with low Ca^{2+} (0.1 mM)-high Mg^{2+} (10 mM) medium containing TTX (0.3 μM) throughout the experiment.

preliminary experiments with older rats (7–10-day-old), we noticed a tendency for the inhibition of slow VRP evoked by NO donors to become less pronounced and for the ventral root depolarization to become more pronounced (unpublished observation). This possible decline of the NO-mediated inhibitory influence may also explain the result of Thompson *et al.* (1995), in which they found that a nociceptive response was not affected by NOS inhibitors and sodium nitroprusside (SNP) in isolated spinal cords of 10–12-day-old rats.

What types of stimulation may evoke NO-mediated spinal antinociception are currently unknown. Of particular interest is that cholinergic (probably muscarinic receptor-mediated) analgesia at the spinal cord has been suggested to be mediated by NO (Zhou *et al.*, 1993; Iwamoto & Marion, 1994). We previously found that some muscarinic agonists, such as arecoline and oxotremorine, selectively inhibit the slow VRPs evoked by either electrical stimulation of a cutaneous nerve or noxious skin stimulation in the neonatal rat spinal cord preparations (Kurihara *et al.*, 1993). Muscarinic agonists have also been shown to stimulate production of NO in capsaicin-sensitive sensory neurones (Bauer *et al.*, 1994). Further study is necessary to clarify the relationships between the muscarinic cholinergic system and NO system in the spinal cord.

The source of endogenous NO and the synaptic sites of its actions remain to be clarified. In the neonatal rat it has been shown that the neuronal NOS staining in the superficial layer of dorsal horn is almost absent in the lumbar spinal cord (see Introduction), whereas at least 20% of neuronal cells in dorsal root ganglia are stained for neuronal NOS at postnatal day 3 (Bredt & Snyder, 1994b). The endogenous NO that causes the inhibitory effect on the slow VRP may therefore be derived from primary afferent terminals to negatively auto-regulate the release of sensory neurotransmitters. However, previous results on the role of NO in the neuropeptide release from primary sensory neurones are conflicting. Garry *et al.* (1994) showed that SNP evoked the release of substance P (SP) and calcitonin gene-related peptide (CGRP) from rat dorsal horn. Dymshitz & Vasko (1994), however, showed that the NO donors, SNAP and SIN-1, and 8-Br-cyclic GMP, did not alter the release of SP and CGRP from rat sensory neurones in culture. Although the latter group observed that SNP enhanced the capsaicin-induced SP and CGRP release, decomposed products of SNP were active as well, suggesting a NO-independent action of SNP.

Other sites that are stained for neuronal NOS around postnatal day 3 in the lumbar spinal cord include deeper laminae of the dorsal horn, lamina X (Liuzzi *et al.*, 1993; Soyguder *et al.*, 1994) and the ventral horn (Kalb & Agostini, 1993). Kalb & Agostini (1993) observed that neuronal NOS staining is transiently expressed in premotor interneurones and suggested that local production of NO in the ventral horn during early postnatal life may contribute to motor neurone

differentiation. The depolarizing response of motoneurones to NO donors observed in this study may be relevant to this phenomenon.

Both the inhibitory action on slow VRPs and the depolarizing action of NO and NO donors were mimicked by 8-Br-cyclic GMP, which suggests that both the actions are mediated by the guanylyl cyclase-cyclic GMP cascade. It is becoming clear that cyclic GMP interacts with several types of intracellular receptor proteins including PKG, cyclic GMP-regulated cyclic nucleotide phosphodiesterases (PDE2 and PDE3 families) and cyclic GMP-regulated ion channels to modulate cellular functions (see Lincoln & Cornwell, 1993; Lincoln, 1995, for reviews). The PKG inhibitor KT5823 partly blocked the inhibitory effects of NOC12, GSNO and 8-Br-cyclic GMP, suggesting that NO-cyclic GMP cascade caused the slow VRP inhibition via both PKG-dependent and independent mechanisms. The reason that the inhibitory effect of SIN-1 was almost completely blocked by KT5823 is unknown. This might be related to the fact that SIN-1 was devoid of depolarizing action. Unlike the inhibitory action, the depolarizing actions of NO donors do not seem to involve activation of PKG, because they were resistant to the PKG inhibitor.

Possible involvement of PDE2s (cyclic GMP-stimulated) and PDE3s (cyclic GMP-inhibited) in the slow VRP inhibition evoked by NO donors appears to be unlikely, since (1) 8-Br-cyclic GMP has been shown to be a very poor activator of PDE2s (Erneux *et al.*, 1981) and (2) although the action of cyclic GMP through the inhibition of PDE3s is supposed to be due to the subsequent elevation of the levels of cyclic AMP (Lincoln & Cornwell, 1993), 8-Br-cyclic AMP was found to have only a weak inhibitory action on the slow VRP. Since 8-Br-cyclic AMP depolarized ventral roots as potent as 8-Br-cyclic GMP, the PDE3 family may be involved in the excitatory response. Whether cyclic GMP-regulated ion channels are involved in the NO-mediated responses, in particular, the excitatory response, remains to be clarified.

The finding that the depolarization of ventral roots evoked by NO donors was markedly augmented by TTX and/or low Ca^{2+}-high Mg^{2+} medium, which was employed to block synaptic transmission, was unexpected. Furthermore, the depolarizing response in the medium was markedly inhibited by the EAA receptor antagonists. The latter observation suggests that the excitatory action of NO is at least partly mediated by release of EAAs. In support of this concept, we observed that SNAP induced release of amino acids (glutamate, aspartate, γ-aminobutyric acid, glycine and taurine) from the isolated neonatal rat spinal cord and the release was not blocked by Ca^{2+}-free medium (Kurihara *et al.*, 1995). The tendency for the depolarizing action of NO to show desensitization may be explained by a depletion of available neurotransmitters in the releasable pool. The mechanism of the Ca^{2+}-independent release of amino acids evoked by NO donors is now under investigation. The significance of the NO-evoked amino acid release is unknown, but the release of EAAs may contribute to the developments of hyperalgesia and allodynia (see Introduction).

Among the NO donors examined, SIN-1 was unique in that it inhibited the slow VRPs without depolarization of ventral roots (Figure 1c). Recently SIN-1 was shown to generate both NO and superoxide anion (O_2^-) almost simultaneously (Feelisch *et al.*, 1989). These two molecules bind rapidly and give peroxynitrite ($ONOO^-$) (Beckman *et al.*, 1990; Saran *et al.*, 1990) and further lead to the production of hydroxyl radical ($HO^\bullet$) (Hogg *et al.*, 1992). This may explain its relatively weak potency, slow onset, and susceptibility to the NO scavenger and the PKG inhibitor. In preliminary experiments we observed that SIN-1 (100–300 μM) depolarized ventral roots in the presence of superoxide dismutase (unpublished observations). This suggests that superoxide anion, peroxynitrite, hydroxy radical or other metabolites may interfere with the depolarizing action of NO, or cause hyperpolarization of ventral roots, which cancels the NO-evoked depolarization. Although carboxy-PTIO considerably reduced the inhibition of slow VRP induced by NOC12 and SNAP, the possibility that peroxynitrite or hydroxyl radical also contribute to the inhibition of slow VRPs remains, since peroxynitrite has been shown to stimulate soluble guanylyl cyclase by releasing NO (Mayer *et al.*, 1995) and hydroxyl radical is known to be one of the endogenous soluble guanylyl cyclase activators (Schmidt, 1992).

We are grateful to Professor Emeritus Masanori Otsuka for his encouragement and critical reading of this manuscript. We also thank Ms Miki Suzuki for her excellent technical assistance. This work was supported by grants-in aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan (Nos. 3612 and 06670102). T.K. is a Research Fellow of the Japan Society for the Promotion of Science.

References

AKAIKE, T., YOSHIDA, M., MIYAMOTO, Y., SATO, K., KOHNO, M., SASAMOTO, K., MIYAZAKI, K., UEDA, S. & MAEDA, H. (1993). Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/•NO through a radical reaction. *Biochemistry*, **32**, 827–832.

AKAGI, H., KONISHI, S., OTSUKA, M. & YANAGISAWA, M. (1985). The role of substance P as a neurotransmitter in the reflexes of slow time courses in the neonatal rat spinal cord. *Br. J. Pharmacol.*, **84**, 663–673.

BAUER, M.B., MURPHY, S. & GEBHART, G.F. (1994). Muscarinic cholinergic stimulation of the nitric oxide-cyclic GMP signaling system in cultured rat sensory neurons. *Neuroscience*, **62**, 351–359.

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydoxy radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BÖHME, G.A., BON, C., STUTZMANN, J.-M., DOBEL, A. & BLANCHARD, J.-C. (1991). Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.*, **199**, 379–381.

BREDT, D.S. & SNYDER, S.H. (1994a). Nitric oxide: A physiologic messenger molecule. *Ann. Rev. Biochem.*, **63**, 175–195.

BREDT, D.S. & SNYDER, S.H. (1994b). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron*, **13**, 301–313.

DAWSON, T.M. & SNYDER, S.H. (1994). Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *J. Neurosci.*, **14**, 5147–5159.

DUN, N.J., DUN, S.L., WU, S.Y., FÖRSTERMANN, U., SCHMIDT, H.H.H.W. & TSENG, L.F. (1993). Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. *Neuroscience*, **54**, 845–857.

DYMSHITZ, J. & VASKO, M.R. (1994). Nitric oxide and cyclic guanosine 3′,5′-monophosphate do not alter neuropeptide release from rat sensory neurons grown in culture. *Neuroscience*, **62**, 1279–1286.

ERNEUX, C., COUCHIE, D., DUMONT, J.E., BARANIAK, J., STEC, W.J., ABBAD, E.G., PETRIDIS, G. & JASTORFF, B. (1981). Specificity of cyclic GMP activation of a multi-substrate cyclic nucleotide phosphodiesterase from rat liver. *Eur. J. Biochem.*, **115**, 503–510.

FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17, (Suppl. 3)**, S25–S33.

FEELISCH, M., OSTROWSKI, J. & NOACK, E. (1989). On the mechanism of NO release from sydnonimines. *J. Cardiovasc. Pharmacol.*, **14, (Suppl. 11)**, S13–S22.

GARRY, M.G., RICHARDSON, J.D. & HARGREAVES, K.M. (1994). Sodium nitroprusside evokes the release of immunoreactive calcitonin gene-related peptide and substance P from dorsal horn slices via nitric oxide-dependent and nitric oxide-independent mechanisms. *J. Neurosci.*, **14**, 4329–4337.

GARTHWAITE, J. & BOULTON, C.L. (1995). Nitric oxide signaling in the central nervous system. *Ann. Rev. Physiol.*, **57**, 683–706.

GUO, J.-Z., YOSHIOKA, K., YANAGISAWA, M., HOSOKI, R., HAGAN, R.M. & OTSUKA, M. (1993). Depression of primary afferent-evoked responses by GR71251 in the isolated spinal cord of the neonatal rat. *Br. J. Pharmacol.*, **110**, 1142–1148.

HALEY, J.E., DICKENSON, A.H. & SCHACHTER, M. (1992). Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. *Neuropharmacol.*, **31**, 251–259.

HOGG, N., DARLEY-USMAR, V.M., WILSON, M.T. & MONCADA, S. (1992). Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.*, **281**, 419–424.

HOSOKI, R., YANAGISAWA, M., GUO, J.-Z., YOSHIOKA, K., MAEHARA, T. & OTSUKA, M. (1994). Effects of RP 67580, a tachykinin NK_1 receptor antagonist, on a primary afferent-evoked response of ventral roots in the neonatal rat spinal cord. *Br. J. Pharmacol.*, **113**, 1141–1146.

IWAMOTO, E.T. & MARION, L. (1994). Pharmacologic evidence that spinal muscarinic analgesia is mediated by an L-arginine/nitric oxide/cyclic GMP cascade in rats. *J. Pharmacol. Exp. Ther.*, **271**, 601–608.

KALB, R.G. & AGOSTINI, J. (1993). Molecular evidence for nitric oxide-mediated motor neuron development. *Neuroscience*, **57**, 1–8.

KITTO, K.F., HALEY, J.E. & WILCOX, G.L. (1992). Involvement of nitric oxide in spinally mediated hyperalgesia in the mouse. *Neurosci. Lett.*, **148**, 1–5.

KREEGER, J.S., KITTO, K.F. & LARSON, A.A. (1994). Substance P N-terminal metabolites and nitric oxide mediate capsaicin-induced antinociception in the adult mouse. *J. Pharmacol. Exp. Ther.*, **271**, 1281–1285.

KURIHARA, T., SUZUKI, H., YANAGISAWA, M. & YOSHIOKA, K. (1993). Muscarinic excitatory and inhibitory mechanisms involved in afferent fibre-evoked depolarization of motoneurones in the neonatal rat spinal cord. *Br. J. Pharmacol.*, **110**, 61–70.

KURIHARA, T. & YOSHIOKA, K. (1994a). Involvement of nitric oxide in a nociceptive response in the neonatal rat spinal cord. *Jpn. J. Pharmacol. Suppl.*, **64**, 173P.

KURIHARA, T. & YOSHIOKA, K. (1994b). Excitatory and inhibitory modulation by nitric oxide of nociceptive transmission in the neonatal rat spinal cord. *Neurosci. Res. Suppl.*, **19**, S77.

KURIHARA, T., SUZUKI, H. & YOSHIOKA, K. (1995). The role of nitric oxide in spinal nociceptive transmission in the neonatal rat. *Fourth IBRO World Congress of Neurosci.*, A5.15 (Abstract).

LINCOLN, T.M. & CORNWELL, T.L. (1993). Intracellular cyclic GMP receptor proteins. *FASEB J.*, **7**, 328–338.

LINCOLN, T.M. (1995). Cyclic GMP receptor proteins: role in nervous system and other tissues. In *Nitric Oxide in the Nervous System*. ed. Vincent, S. pp. 51–81. London: Academic Press.

LIUZZI, F.J., WU, W., SCOVILLE, S.A. & SCHINCO, F.P. (1993). Development of nitric oxide synthase expression in the superficial dorsal horn of the rat spinal cord. *Exp. Neurol.*, **121**, 275–278.

LOTHE, A., LI, P., TONG, C., YOON, Y., BOUAZIZ, H., DETWEILER, D. & EISENACH, J.C. (1994). Spinal cholinergic *alpha*-2 adrenergic interaction in analgesia and hemodynamic control: role of muscarinic receptor subtypes and nitric oxide. *J. Pharmacol. Exp. Ther.*, **270**, 1301–1306.

MALMBERG, A.B. & YAKSH, T.L. (1993). Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. *Pain*, **54**, 291–300.

MAYER, B., SCHRAMMEL, A., KLATT, P., KOESLING, D. & SCHMIDT, K. (1995). Peroxynitrite-induced accumulation of cyclic GMP in endothelial cells and stimulation of purified soluble guanylyl cyclase. Dependence on glutathione and possible role of S-nitrosation. *J. Biol. Chem.*, **270**, 17355–17360.

MCMAHON, S.B., LEWIN, G.R. & WALL, P.D. (1993). Central hyperexcitability triggered by noxious inputs. *Curr. Opin. Neurobiol.*, **3**, 602–610.

MELLER, S.T., DYKSTRA, C. & GEBHART, G.F. (1992a). Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex. *Eur. J. Pharmacol.*, **214**, 93–96.

MELLER, S.T. & GEBHART, G.F. (1993). Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain*, **52**, 127–136.

MELLER, S.T., PECHMAN, P.S., GEBHART, G.F. & MAVES, T.J. (1992b). Nitric oxide mediates the thermal hyperalgesia produced in a model of neuropathic pain in the rat. *Neuroscience*, **50**, 7–10.

MONCADA, S. & HIGGS, A. (1993). The L-arginine-nitric oxide pathway. *New Eng. J. Med.*, **329**, 2002–2012.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G-nitro arginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.*, **102**, 198–202.

NAKANISHI, S. (1989). K-252 derivatives-K-252a, K-252b, KT5720, KT5926, KT5823. *Seitaino-Kagaku*, **40**, 364–365 (in Japanese).

NUSSBAUMER, J.-C., YANAGISAWA, M. & OTSUKA, M. (1989). Pharmacological properties of a C-fibre response evoked by saphenous nerve stimulation in an isolated spinal cord-nerve preparation of the newborn rat. *Br. J. Pharmacol.*, **98**, 373–382.

O'DELL, T.J., HAWKINS, R.D., KANDEL, E.R. & ARANCIO, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11285–11289.

OTSUKA, M. & YANAGISAWA, M. (1987). Does substance P act as a pain transmitter? *Trends Pharmacol. Sci.*, **8**, 506–510.

OTSUKA, M. & YANAGISAWA, M. (1988). Effect of a tachykinin antagonist on a nociceptive reflex in the isolated spinal cord-tail preparation of the newborn rat. *J. Physiol.*, **395**, 255–270.

SAITO, S., KIDD, G.J., TRAPP, B.D., DAWSON, T.M., BREDT, D.S., WILSON, D.A., TRAYSTMAN, R.J., SNYDER, S.H. & HANLEY, D.F. (1994). Rat spinal cord neurons contain nitric oxide synthase. *Neuroscience*, **59**, 447–456.

SARAN, M., MICHEL, C. & BORS, W. (1990). Reaction of NO with O_2^-. Implications for the action of endothelium-derived relaxing factor (EDRF). *Free Radical Res. Commun.*, **10**, 221–226.

SCHMIDT, H.H.H.W. (1992). NO$^\bullet$, CO and $^\bullet$OH. Endogenous soluble guanylyl cyclase-activating factors. *FEBS Lett.*, **307**, 102–107.

SCHMIDT, H.H.H.W., LOHMANN, S.M. & WALTER, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochem. Biophys. Acta*, **1178**, 153–175.

SCHUMAN, E.M. (1995). Nitric oxide signaling, long-term potentiation and long-term depression. In *Nitric Oxide in the Nervous System*. ed. Vincent, S. pp. 125–150. London: Academic Press.

SCHUMAN, E.M. & MADISON, D.V. (1994). Nitric oxide and synaptic function. *Ann. Rev. Neurosci.*, **17**, 153–183.

SCHUMAN, E.M. & MADISON, D.V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science*, **254**, 1503–1506.

SHIBUKI, K. & OKADA, D. (1991). Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature*, **349**, 326–328.

SOYGUDER, Z, SCHMIDT, H.H.H.W. & MORRIS, R. (1994). Postnatal development of nitric oxide synthase type 1 expression in the lumbar spinal cord of the rat: a comparison with the induction of c-*fos* in response to peripheral application of mustard oil. *Neurosci. Lett.*, **180**, 188–192.

SPIKE, R.C., TODD, A.J. & JOHNSTON, H.M. (1993). Coexistence of NADPH diaphorase with GABA, glycine, and acetylcholine in rat spinal cord. *J. Comp. Neurol.*, **335**, 320–333.

TAKAGI, H., HARIMA, A. & SHIMIZU, H. (1990). A novel clinical treatment of persistent pain with L-arginine. *Eur. J. Pharmacol.*, **183**, 1443.

THOMPSON, S.W.N., BABBEDGE, R., LEVERS, T., DRAY, A. & URBAN, L. (1995). No evidence for contribution of nitric oxide to spinal reflex activity in the rat spinal cord in vitro. *Neurosci. Lett.*, **188**, 121–124.

VALTSCHANOFF, J.G., WEINBERG, R.J. & RUSTIONI, A. (1992a). NADPH diaphorase in the spinal cord of rats. *J. Comp. Neurol.*, **321**, 209–222.

VALTSCHANOFF, J.G., WEINBERG, R.J., RUSTIONI, A. & SCHMIDT, H.H.H.W. (1992b). Nitric oxide synthase and GABA colocalize in lamina II of rat spinal cord. *Neurosci. Lett.*, **148,** 6–10.

VINCENT, S.R. (1994). Nitric oxide: a radical neurotransmitter in the central nervous system. *Prog. Neurobiol.*, **42,** 129–160.

YANAGISAWA, M., HOSOKI, R. & OTSUKA, M. (1992). The isolated spinal cord-skin preparation of the newborn rat and effects of some algogenic and analgesic substances. *Eur. J. Pharmacol.*, **220,** 111–117.

ZHANG, X., VERGE, V., WIESENFELD-HALLIN, Z., JU, G., BREDT, D., SNYDER, S.H. & HÖKFELT, T. (1993). Nitric oxide synthase-like immunoreactivity in lumbar dorsal root ganglia and spinal cord of rat and monkey and effect of peripheral axotomy. *J. Comp. Neurol.*, **335,** 563–575.

ZHUO, M., MELLER, S.T. & GEBHART, G.F. (1993). Endogenous nitric oxide is required for tonic cholinergic inhibition of spinal mechanical transmission. *Pain*, **54,** 71–78.

(Received January 12, 1996
Revised March 29, 1996
Accepted April 4, 1996)