Nitric Oxide Inhibits Spinally Projecting Paraventricular Neurons Through Potentiation of Presynaptic GABA Release

DE-PEI LI,1 SHAO-RUI CHEN,1 AND HUI-LIN PAN1,2
1Department of Anesthesiology and 2Department of Neuroscience and Anatomy, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Received 24 July 2002; accepted in final form 1 August 2002

Li, De-Pei, Chen, Shao-Rui and Hui-Lin Pan. Nitric oxide inhibits spinally projecting paraventricular neurons through potentiation of presynaptic GABA release. J Neurophysiol 88: 2664–2674, 2002; 10.1152/jn.00540.2002. Nitric oxide (NO) in the paraventricular nucleus (PVN) is involved in the regulation of the excitability of PVN neurons. However, the effect of NO on the inhibitory GABAergic and excitatory glutamatergic inputs to spinally projecting PVN neurons has not been studied specifically. In the present study, we determined the role of the inhibitory GABAergic and excitatory glutamatergic inputs in the inhibitory action of NO on spinally projecting PVN neurons. Spinally projecting PVN neurons were retrogradely labeled by a fluorescent dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI), injected into the spinal cord of rats. Whole cell voltage- and current-clamp recordings were performed on DiI-labeled PVN neurons in the hypothalamic slice. The spontaneous miniature inhibitory postsynaptic currents (mIPSCs) recorded in DiI-labeled neurons were abolished by 20 μM bicuculline, whereas the miniature excitatory postsynaptic currents (mEPSCs) were eliminated by 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione. Bath application of an NO donor, 100 μM S-nitroso-N-acetyl-penicillamine (SNAP), or the NO precursor, 100 μM l-arginine, both significantly increased the frequency of mIPSCs of DiI-labeled PVN neurons, without altering the amplitude and the decay time constant of mIPSCs. The effect of SNAP and l-arginine on the frequency of mIPSCs was eliminated by application of 20 μM M bicuculline, whereas the miniature excitatory postsynaptic currents (mEPSCs) were eliminated by 20 μM M bicuculline from the PVN neurons. The spontaneous miniature inhibitory postsynaptic currents (mIPSCs) recorded in DiI-labeled neurons were abolished by 20 μM bicuculline, whereas the miniature excitatory postsynaptic currents (mEPSCs) were eliminated by 20 μM M bicuculline from the PVN neurons. The spontaneous miniature inhibitory postsynaptic currents (mIPSCs) recorded in DiI-labeled neurons were abolished by 20 μM bicuculline, whereas the miniature excitatory postsynaptic currents (mEPSCs) were eliminated by 20 μM M bicuculline from the PVN neurons. For instance, PVN neurons project to cardiovascular centers in the medulla such as the rostral ventrolateral medulla (RVLM) and nucleus of the solitary tract as well as sympathetic preganglionic neurons located in the intermediolateral (IML) cell column of the spinal cord (Hardy 2001; Pyner and Coote 2000; Ranson et al. 1998; Yamashita et al. 1984). The PVN-IML pathway is especially important in regulation of the hemodynamic responses to stress and osmolarity changes in the blood (Coote 1995; Imaki et al. 1998; Swanson and Sawchenko 1983). However, the synaptic mechanisms involved in regulation of the excitability of spinally projecting PVN neurons are not fully known.

As a gaseous, non-conventional neurotransmitter in the central nervous system, nitric oxide (NO) plays an important role in regulation of sympathetic outflow in the CNS (Krukkoff 1999). In this regard, intracerebroventricular injection of NO precursors or microinjection of sodium nitroprusside (SNP) into the PVN reduces blood pressure and sympathetic nerve activity, and such an effect can be blocked by the NO synthase inhibitor (Nishimura et al. 1997; Zhang and Patel 1998; Zhang et al. 1997). Also, the neuronal NO synthase (nNOS) is expressed in the PVN, suggesting that endogenous NO may be involved in the regulation of the endocrine and sympathetic nervous systems (Arevalo et al. 1992; Hatakeyama et al. 1996; Nylen et al. 2001). A majority of the synaptic inputs to the PVN originate from the suprachiasmatic nucleus, subfornical organ, and local sources (Anderson et al. 2001; Bains and Ferguson 1995; Boudaba et al. 1996; Cui et al. 2001; Hermes et al. 1996; Tasker and Dudek 1993). The GABA synaptic inputs make up ~50% of the synaptic innervation of PVN neurons (Decavel and Van den Pol 1990). The electrophysiological studies have further demonstrated that the majority of the local synaptic inputs to PVN neurons are GABAergic (Boudaba et al. 1996; Tasker and Dudek 1993). Consistent with the above findings, microinjection of bicuculline into the PVN in conscious rats increases blood pressure and heart rate (Schlenker et al. 2001).

The interaction of NO and GABA within the PVN in the control of sympathetic outflow has been suggested in previous studies. For instance, the sympathetic inhibitory effect produced by microinjection of SNP into the PVN is eliminated by bicuculline (Zhang and Patel 1998). Also, perfusion with NO-

INTRODUCTION

The paraventricular nucleus (PVN) of the hypothalamus is an important site for regulation of various neuroendocrine and autonomic functions (Cui et al. 2001; Imaki et al. 1998; Pyner and Coote 1999; Swanson and Sawchenko 1980, 1983). For instance, PVN neurons project to cardiovascular centers in the medulla such as the rostral ventrolateral medulla (RVLM) and nucleus of the solitary tract as well as sympathetic preganglionic neurons located in the intermediolateral (IML) cell column of the spinal cord (Hardy 2001; Pyner and Coote 2000; Ranson et al. 1998; Yamashita et al. 1984). The PVN-IML pathway is especially important in regulation of the hemodynamic responses to stress and osmolarity changes in the blood (Coote 1995; Imaki et al. 1998; Swanson and Sawchenko 1983). However, the synaptic mechanisms involved in regulation of the excitability of spinally projecting PVN neurons are not fully known.

As a gaseous, non-conventional neurotransmitter in the central nervous system, nitric oxide (NO) plays an important role in regulation of sympathetic outflow in the CNS (Krukkoff 1999). In this regard, intracerebroventricular injection of NO precursors or microinjection of sodium nitroprusside (SNP) into the PVN reduces blood pressure and sympathetic nerve activity, and such an effect can be blocked by the NO synthase inhibitor (Nishimura et al. 1997; Zhang and Patel 1998; Zhang et al. 1997). Also, the neuronal NO synthase (nNOS) is expressed in the PVN, suggesting that endogenous NO may be involved in the regulation of the endocrine and sympathetic nervous systems (Arevalo et al. 1992; Hatakeyama et al. 1996; Nylen et al. 2001). A majority of the synaptic inputs to the PVN originate from the suprachiasmatic nucleus, subfornical organ, and local sources (Anderson et al. 2001; Bains and Ferguson 1995; Boudaba et al. 1996; Cui et al. 2001; Hermes et al. 1996; Tasker and Dudek 1993). The GABA synaptic inputs make up ~50% of the synaptic innervation of PVN neurons (Decavel and Van den Pol 1990). The electrophysiological studies have further demonstrated that the majority of the local synaptic inputs to PVN neurons are GABAergic (Boudaba et al. 1996; Tasker and Dudek 1993). Consistent with the above findings, microinjection of bicuculline into the PVN in conscious rats increases blood pressure and heart rate (Schlenker et al. 2001).

The interaction of NO and GABA within the PVN in the control of sympathetic outflow has been suggested in previous studies. For instance, the sympathetic inhibitory effect produced by microinjection of SNP into the PVN is eliminated by bicuculline (Zhang and Patel 1998). Also, perfusion with NO-
containing artificial cerebrospinal fluid causes a significant GABA increase within the PVN (Horn et al. 1994). These observations raise the possibility that the inhibitory effect of NO on the sympathetic outflow is likely mediated by the modulation of synaptic GABA release onto spinally projecting PVN neurons. However, the effect of NO on GABAergic and glutamatergic synaptic inputs to spinally projecting PVN neurons has not been studied specifically. Therefore, in this study, we used a combination of retrograde labeling and in vitro whole cell recording techniques in the hypothalamic slice to determine the effect of NO on the inhibitory GABAergic and excitatory glutamatergic inputs to spinally projecting PVN neurons. The role of GABAergic synaptic inputs in the inhibitory action of NO on the firing activity of spinally projecting PVN neurons was also investigated.

METHODS

Retrograde labeling of spinally projecting PVN neurons

Sprague-Dawley rats (3–5 wk old, Harlan, Indianapolis, IN) of either sex were used for this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Penn State University College of Medicine and conformed to the National Institutes of Health guidelines on the ethical use of animals. All efforts were made to minimize both the suffering and number of animals used. The rat spinal cord at the T1–T4 level was exposed through dorsal laminectomy under halothane anesthesia. The fluorescence tracer, 1,1’-dioctadecyl-3,3,3,3’-tetramethylindocarbocyanine (DiI, Molecular Probes, Eugene, OR), was dissolved in DMSO (10–15 mg dissolved in 200 μl of DMSO) and was pressure-injected (Picospritzer II, General Valve, Fairfield, NJ) bilaterally into the region of the IML column of the spinal cord in 1 or 2 100-nl injections using a glass micropipette (20–30 μm tip diam.). The pipette was positioned with a micromanipulator at ~500 μm below the dorsolateral sulcus, and the injection of DiI was monitored through a surgical microscope. DiI was chosen because this tracer has been used in a previous study and is devoid of toxicity to neurons (Kangrga and Loewy 1994). After injection, the muscles were sutured and the wound was closed. Animals were returned to their cages for 3–7 days, which is sufficient time to permit retrograde tracer transport to the PVN (Kangrga and Loewy 1994).

Slice preparations

A total of 30 rats were used for the electrophysiology experiments. Three to 7 days after DiI injection, the rats were rapidly decapitated under halothane anesthesia. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid perfusion solution saturated with 95% O2–5% CO2 for 1–2 min. A tissue block containing the hypothalamus was cut from the brain and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO) as described previously (Li and Pan 2001; Li et al. 2001a). Coronal slices (300 μm in thickness) containing the PVN were cut from the tissue block at 4°C. The slices were pre-incubated in the artificial cerebral spinal fluid, which was continuously gassed with 95% O2–5% CO2 at 34°C for 1 h until they were transferred to the recording chamber. The perfusion solution contained (in mM) 124.0 NaCl, 3.0 KCl, 1.3 MgSO4, 2.4 CaCl2, 1.4 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3. All the drugs were prepared immediately before the experiments and applied to the slice chamber using syringe pumps.

Recordings of postsynaptic currents of PVN neurons

Recordings of miniature postsynaptic currents were performed in a radio frequency-shielded room using the whole cell voltage-clamp technique as described previously (Li and Pan 2001; Li et al. 2001a). The patch pipettes were triple-pulled (Sutter Instrument, Novato, CA) using boro silicate thin-wall capillaries (1.2 mm OD, 0.86 mm ID; World Precision Instruments, Sarasota, FL). The resistance of the pipette was 4–8 MΩ when it was filled with a solution containing (in mM) 130.0 potassium gluconate, 1.0 MgCl2, 10.0 HEPES, 10.0 EGTA, 1.0 CaCl2, and 4.0 ATP-Mg; adjusted to pH 7.25 with 1M KOH (290–320 mOsm). The slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The slice was perfused at 3.0 ml/min at 34°C maintained by an in-line solution heater and a temperature controller (model TC-324, Warner Instruments). It took 1.5–2 min to completely exchange the solution inside the recording chamber at the perfusion rate of 3 ml/min. To label recorded neurons, biocytin (0.2%) was added into the internal pipette solution. Whole cell recordings from DiI-labeled PVN neurons were made under visual control using a combination of epifluorescence illumination and infrared and differential interference contrast (IR-DIC) optics on an upright microscope (BX50 WI, Olympus, Japan). The DiI-labeled neurons located in the medial third of the PVN area between the third ventricle and the fornix were selected for recording. DiI-labeled neurons were briefly identified with the aid of epifluorescence illumination. The tissue image was captured and enhanced through a camera and displayed on a video monitor. A tight giant-ohm seal was subsequently obtained in the labeled neuron viewed using IR-DIC optics. Recordings of postsynaptic currents began 5 min later after the whole cell access was established and the current reached a steady state. The input resistance was monitored, and the recording was abandoned if it changed >15% (Li and Pan 2001). Recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). A liquid junction potential of −15 mV (for the potassium gluconate pipette solution) was corrected during off-line analysis. Signals were filtered at 1–2 kHz, digitized at 10 kHz using Digidata 1320A (Axon Instruments), and saved to a hard drive of a computer. The miniature inhibitory postsynaptic currents (mIPSCs) and miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 1 μM tetrodotoxin at a holding potential of 0 and −70 mV, respectively (Chiou and Huang 1999; Li and Pan 2001; Li et al. 2001a). Using a similar internal pipette solution, it has been shown that at a holding potential of −70 mV, only mEPSCs are recorded as downward deflections. On the other hand, at a holding potential of 0 mV, only mIPSCs are recorded as upward deflections (Baba et al. 2000; Kabushima et al. 1997).

Recording of spontaneous action potentials of DiI-labeled PVN neurons

The spontaneous action potentials were recorded in PVN neurons using the whole cell current-clamp technique (Chiou and Huang 1999). The recording procedures were similar to those used for postsynaptic current recordings as described in the preceding text except that tetrodotoxin was not used. Recordings of the firing activity of DiI-labeled PVN neurons began ~5 min after the whole cell access was established and the potential reached a steady state. Signals were processed, recorded, and analyzed as described in the preceding text.

To ensure that the recorded neurons were located in the PVN, biocytin (0.2%) was added into the internal pipette solution. After recordings, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for several days. The slice was then cut at a thickness of 35 μm on a freezing microtome (Leica, Germany). Slices were stained with the avidin-biotinylated peroxidase complex (ABC) method (Horikawa and Armstrong 1988; Li and Pan 2001). Briefly, sections were pretreated with 0.2% H2O2 in phosphate buffer to remove the endogenous peroxidase activity. Following incubation in ABC solution (Vector Laboratories, Burlingame, CA) diluted 1:100 in phosphate buffer, sections were reacted with diamobenzidine tetrahydrochloride in the presence of H2O2 (0.003%) and nickel ammonium sulphate (0.05%) for 3–6 min. The sections then were...
mounted on gelatin-coated slides, dried, dehydrated, and coverslipped. The stained cell was identified under a light microscope.

**Immunocytochemistry staining of nNOS in the PVN**

In three separate rats, we determined the spatial relationship of the neuronal NOS (nNOS) positive cells and the DiI-labeled neurons in the PVN. Using halothane anaesthesia, the brain tissue containing the PVN was quickly removed and fixed by submersion in 4% paraformaldehyde for 2–6 days. The sections were then cut to 35 μm in thickness on a freezing microtome and collected free floating in 0.1 M phosphate buffer. Sections were incubated with the primary antibody (rabbit anti-nNOS polyclonal antibody, dilution 1:200, Biomol Research Laboratories, Plymouth Meeting, PA) diluted in Tris-buffered saline (TBS) containing 1% normal goat serum for 2 hours at room temperature and overnight in 4°C. Subsequently, sections were rinsed in TBS and incubated with the secondary antibody (goat anti-rabbit IgG conjugated to Alexa fluor 488, dilution 1:400, Molecular Probe, Eugene, OR) diluted in TBS containing 2% normal goat serum for 1.5 h at room temperature. Then sections were rinsed in TBS for 40 min and mounted on slides, dried, and coverslipped. Negative control was performed by replacing the primary antibody with nonimmune serum from the same species. The sections were viewed using a confocal microscope (Zeiss), and the areas of interest were photographed. Confocal laser scanning microscopy was used for accurate co-localization of fluorescent markers because the thin optical section generated by the confocal microscope eliminates the confounding effects of out-of-focus fluorescence. In each rat, at least four sections from the caudal portion of the PVN were selected and photodocumented for the presence of DiI (red) and nNOS immunoactivity (green). Digital images were adjusted for brightness and contrast using Photoshop 5.0 and then digitally merged. In the higher-magnification images, the co-localization was indicated by the color change (yellow) and represents co-localization, because the optical section thickness (<1 μm) of a confocal image is thin enough to minimize the possibility of superimposition of stained neurons. The number of nNOS positive and DiI-labeled cells and the percentage of those cells double labeled with nNOS and DiI in the PVN was calculated.

**Data analysis**

Data are presented as means ± SE. The mIPSCs, mEPSCs, and the spontaneous action potentials were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). As we have described previously (Li and Pan 2001; Li et al. 2001a), detection of events was accomplished by setting a threshold above the level of the noise. Artifacts in the recording were removed manually. The cumulative probability of the amplitude and inter-event interval of mIPSCs/mEPSCs was compared using the Komogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. At least 100 mIPSCs and mEPSCs were used in each analysis. The effects of drugs on the amplitude and frequency of mIPSCs and mEPSCs were determined by the nonparametric (Wilcoxon signed rank) test or nonparametric ANOVA (Kruskal-Wallis) test with Dunn’s post hoc test. \( P < 0.05 \) was considered to be statistically significant.

**RESULTS**

Whole cell patch-clamp recordings were obtained from 57 PVN cells labeled by DiI. The spinal cord was taken out after sacrificing the rat to verify the injection and diffusion site of DiI in the thoracic spinal cord. The spinal cord slices were viewed under a microscope equipped with fluorescence illumination. The injection site of DiI was largely located in and around the IML in the spinal cord (data not shown). The diffusion size of DiI by examining the spread of DiI around the site of injection was ~0.5 mm in diameter. Figure 1 shows a DiI-labeled PVN neuron identified initially with fluorescence illumination (rhodamine filter, Fig. 1A) and subsequently recorded using differential interference contrast optics (Fig. 1B). The location of all recovered neurons labeled with biocytin was confirmed histologically in the PVN following staining (Fig. 1C). The DiI-labeled PVN neurons displayed a resting membrane potential of ~66.2 ± 4.4 mV (from ~74.5 to ~60.0 mV), an input resistance of 531.6 ± 18.4 MΩ (from 380 to 570 MΩ), and an amplitude of action potentials >60 mV.

**Co-localization of nNOS-positive neurons and DiI-labeled neurons in PVN**

To determine the spatial relationship between nNOS-positive and DiI-labeled neurons in the PVN, the slice containing DiI-labeled PVN neurons was immunostained with a specific nNOS antibody. All negative controls displayed no detectable staining. The distribution patterns of DiI- and nNOS-positive cells in the PVN are shown in Fig. 2. Both nNOS positive cells (green) and the DiI-labeled cells (red) were present in the PVN (Fig. 2). In all sections examined with a high magnification of confocal images, a total of 814 cells were found to be nNOS positive, and 729 cells were labeled with DiI in the PVN. Although many nNOS positive neurons were juxtaposed to the DiI-labeled cells in the PVN, only 46 of 729 (6.3%) DiI-labeled neurons were identified to be nNOS positive (yellow, Fig. 2C).

**Effect of NO on GABAergic mIPSCs in DiI-labeled PVN neurons**

To test the effect of NO on synaptic GABA release onto DiI-labeled PVN neurons, an NO donor, S-nitroso-N-acetylpenicillamine (SNAP), and the NO precursor, L-arginine, were used. The spontaneous mIPSCs were completely abolished by bath application of 20 μM baclofen (n = 12), the antagonist of GABA A receptors (Fig. 3A). The effective concentrations of SNAP and L-arginine were determined in previous studies (Bains and Ferguson 1997b; Ozaki et al. 2000) and our pilot experiments. SNAP, in a concentration of 100 μM, significantly increased the frequency of mIPSCs from 2.75 ± 0.47 to 5.18 ± 0.89 Hz (P < 0.05) without affecting the amplitude and the decay time constant of mIPSCs in all nine neurons tested (Fig. 3A–F). The cumulative probability analysis of mIPSCs before and during SNAP application revealed that the distribution pattern of the inter-event interval of mIPSCs shifted toward the left in response to SNAP, while the distribution pattern of the amplitude was not significantly changed (Fig. 3B). The effect of SNAP on mIPSCs was further analyzed by measuring the time constant of the decay phase of the spontaneous mIPSCs. The decay kinetics of mIPSCs displayed two components, and the decay phase of mIPSCs was best fitted by a double exponential function (Fig. 3D). Neither fast (4.43 ± 0.22 vs. 4.39 ± 0.19 ms) nor slow (20.15 ± 1.34 vs. 19.81 ± 1.41 ms, n = 9) components of the decay phase of mIPSCs during SNAP application was significantly different from those during the control. Similar to the effect of SNAP, we also found that l-arginine, in a concentration of 100 μM, selectively...
potentiated the frequency of spontaneous mIPSCs from 2.41 ± 0.37 to 4.38 ± 0.64 Hz in another nine cells (P < 0.05, Fig. 4 A–D). The amplitude and the decay time constant of mIPSCs remained virtually unaffected by l-arginine. Repeat application of SNAP and l-arginine had a reproducible inhibitory effect on the frequency of mIPSCs (data not shown). In the presence of 20 μM bicuculline, both 100 μM SNAP and 100 μM l-arginine failed to evoke mIPSCs in five PVN neurons tested.

To determine whether the effect of SNAP on mIPSCs was mediated through NO release, a specific NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), was employed. In eight cells, after testing the initial effect of SNAP (100 μM) on mIPSCs, carboxy-PTIO (1 μM) was perfused into the slice chamber. Subsequent application of SNAP failed to increase the frequency of mIPSCs in the presence of carboxy-PTIO (Fig. 5 A–D). Carboxy-PTIO alone had no effect on mIPSCs of PVN neurons in the slice.

FIG. 1. Identification of a retrogradely labeled spinally projecting paraventricular nucleus (PVN) neuron. A: 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled PVN neuron in the slice viewed with fluorescence illumination. B: photomicrograph of the same neuron (*) shown in A with an attached recording electrode (√) in the slice viewed with infrared and differential interference contrast optics. Scale bar = 50 μm in A and B. C: photomicrograph showing the morphology of a recorded DiI-labeled PVN neuron labeled with biocytin. Scale bar = 100 μm. 3V: third ventricle.

FIG. 2. Confocal images showing the spatial relationship of DiI-labeled neurons and neuronal nitric oxide synthase (nNOS)-positive neurons in PVN. A: DiI-labeled PVN neurons (red). B: nNOS immunoreactive neurons (green). C: digitally merged images from A and B. Note that a few DiI-labeled neurons were nNOS positive (yellow). Magnification, ×400. Images are in all cases single confocal optical sections.
Similarly, the effect of L-arginine on mIPSCs was diminished by a specific nNOS inhibitor, 1-(2-trifluoromethylphenyl) imidazole (TRIM, 50 μM; Fig. 6A–D). We found that perfusion of TRIM alone had no effect on mIPSCs. L-arginine failed to increase the frequency of mIPSCs of PVN neurons following treatment of the hypothalamic slice with TRIM in an additional eight neurons (Fig. 6A–D).

Effect of NO on mEPSCs in DiI-labeled PVN neurons

PVN neurons receive both GABAergic and glutamatergic synaptic inputs. To examine the influence of NO on glutamatergic mEPSCs in DiI-labeled PVN neurons, SNAP (100 μM) and L-arginine (100 μM) were used. In nine cells tested, the spontaneous mEPSCs of neurons were eliminated by application of an antagonist of non-N-methyl-D-aspartate (NMDA) glutamate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM, CNQX, Fig. 7A). SNAP had no significant effect on the frequency and amplitude of mEPSCs in six PVN neurons (Fig. 7A–F). The effect of SNAP on mEPSCs was further analyzed by measuring the time constant of the decay phase of mEPSCs. The decay time constant of mEPSCs was best fitted with a single-exponential function (Fig. 7D). The decay time constant was similar during control and during SNAP application (2.36 ± 0.13 vs. 2.41 ± 0.19 ms, n = 6). In another six neurons, L-arginine also failed to alter on the frequency and amplitude of mEPSCs (data not shown).

Effect of NO on the excitability of DiI-labeled PVN neurons

Because NO increases the inhibitory GABAergic input to DiI-labeled PVN neurons, it is possible that the excitability of these neurons would be inhibited by NO. To directly test this hypothesis, the effect of SNAP or L-arginine on the firing activity of DiI-labeled PVN neurons was determined using whole cell current-clamp recordings. The majority of DiI-labeled PVN neurons recorded (12 of 15) displayed spontaneous discharge activity. Both SNAP and L-arginine significantly
inhibited the firing activity of PVN neurons (Figs. 8–10). SNAP, in a concentration of 100 μM, significantly decreased the discharge rate of PVN neurons from 5.09 ± 0.62 to 0.44 ± 0.09 Hz in six cells tested (P < 0.05, Figs. 8 and 10). Application of SNAP (100 μM) only slightly increased the resting membrane potential (−70.3 ± 1.47 to −67.8 ± 1.49 mV, P > 0.05). Similar to the effect of SNAP, l-arginine (100 μM) also significantly decreased the discharge frequency in another six cells tested (from 4.39 ± 0.36 to 0.39 ± 0.10 Hz, P < 0.05, Figs. 9 and 10) without significantly altering the resting membrane potential (−69.6 ± 2.4 to −66.2 ± 3.6 mV, P > 0.05).

Role of GABA<sub>A</sub> receptors in NO-induced inhibition on Dil-labeled PVN neurons

To determine the role of the GABAergic synaptic input and GABA<sub>A</sub> receptors in the inhibitory effect of NO on Dil-labeled PVN neurons, the effect of SNAP or l-arginine on the firing activity of Dil-labeled PVN neurons was determined in the presence of a GABA<sub>A</sub> receptor antagonist, bicuculline. The spontaneous discharge activity of 12 neurons was significantly increased following perfusion of 20 μM bicuculline (Figs. 9 and 10). Subsequent application of 100 μM l-arginine or 100 μM SNAP failed to inhibit the spontaneous activity of neurons in the presence of 20 μM bicuculline (Figs. 9 and 10). Figure 10 summarizes the effects of SNAP (100 μM, n = 6) and l-arginine (100 μM, n = 6) on the discharge frequency of neurons in the presence of 20 μM bicuculline.

FIG. 4. Effect of l-arginine on mIPSCs of Dil-labeled PVN neurons. A: representative tracings showing changes of mIPSCs during control, perfusion of l-arginine (100 μM), washout, and bicuculline (20 μM). B: inter-event interval histograms showing the distribution patterns during control, l-arginine application, and washout. l-arginine increased the number of mIPSCs in the short-interval range, indicating that the frequency of mIPSCs increased during administration of l-arginine (P < 0.05, Kolmogorov-Smirnov test). C and D: summary data showing the effect of l-arginine on the frequency (C) and the amplitude (D) of mIPSCs of 9 Dil-labeled PVN neurons. Data presented as means ± SE. *P < 0.05 compared to the control (Kruskal-Wallis test).

DISCUSSION

This is the first electrophysiological study examining the potential influence of NO on excitatory and inhibitory synaptic inputs to spinally projecting PVN neurons. We found that an NO donor, SNAP, or the NO precursor, l-arginine, significantly increased the frequency of GABAergic mIPSCs in spinally projecting PVN neurons, without affecting the amplitude and the decay time constant of mIPSCs. The NO-induced potentiation of mIPSCs was eliminated by application of an NO scavenger, carboxy-PTIO, or a specific nNOS inhibitor, TRIM. On the other hand, SNAP and l-arginine had no effect on mEPSCs recorded from spinally projecting PVN neurons.
Furthermore, we observed that both SNAP and l-arginine significantly inhibited the discharge activity of spinally projecting PVN neurons, and this inhibitory effect was abolished by pre-treatment with bicuculline, a GABA_A receptor antagonist. Therefore the present study provides substantial evidence that NO inhibits the excitability of spinally projecting PVN neurons through augmentation of the inhibitory GABAergic synaptic input.

In the present study, we used the retrograde labeling technique to identify the PVN neurons projecting to the spinal cord to specifically study this descending pathway related to the control of sympathetic outflow. An intrinsic limitation of this technique is that only neurons relatively close to the surface, an area that is more prone to damage during slice preparation, can be visualized (Kangrga and Loewy 1994). Thus structural preservation is not always optimal. Damaged (e.g., somatic swelling, missing, or cut dendritic trees) or weakly labeled neurons were not sampled for recordings in our study. It should be acknowledged that PVN neurons also innervate the dorsal horn of the spinal cord (Swanson and McKellar 1979). The large injection volume of Dil can spread to regions outside of the IML. As a result, we cannot exclude the possibility that some of the Dil-labeled PVN neurons may project to the dorsal horn of the spinal cord. We found that the input resistance of spinally projecting PVN cells recorded from the present study was significantly lower than that of magnocellular neurons or some parvocellular neurons in the PVN. This feature is similar to that reported in a recent study (Cui et al. 2001), suggesting that spinally projecting PVN cells represent a subpopulation of parvocellular neurons. Consistent with previous studies (Arevalo et al. 1992; Hatakeyama et al. 1996; Nylen et al. 2001), we found that densely stained nNOS neurons were present extensively in the PVN and were in close contact with Dil-labeled neurons. However, we observed that only 6.3% of Dil-labeled PVN neurons were nNOS positive. This observation is similar to a previous study showing that a few NOS-positive neurons

**FIG. 5.** Effect of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) plus SNAP on mIPSCs in Dil-labeled PVN neurons. A: histogram showing the frequency of mIPSCs in response to perfusion of SNAP (100 μM) and SNAP plus carboxy-PTIO. Note that pre-treatment with carboxy-PTIO (1 μM) abolished the SNAP-induced increase in frequency of mIPSCs. B: plot of the amplitude of mIPSCs as a function of time for the same Dil-labeled PVN neuron shown in A. Each point represents averaged values of mIPSCs during a period of 30 s. C: original tracings of mIPSCs in the same Dil-labeled neuron obtained during control, application of SNAP, and SNAP plus carboxy-PTIO. D and E: summary data showing the effect of SNAP and SNAP plus carboxy-PTIO on mIPSCs of 8 Dil-labeled PVN neurons. *P < 0.05 compared to the control (Wilcoxon signed-rank test).
project to the spinal cord using NADPH-diaphorase as a marker for NOS-containing cells (Hatakeyama et al. 1996). Although most of the spinally projecting neurons included in our study likely are not nNOS-containing neurons, it is important to note that the cells studied could be influenced by NO produced from the neighboring PVN neurons since NO can diffuse from its site of production and affect relatively distant neurons.

In the present study, we further investigated the effect of NO and its interaction with the GABAergic synaptic input in the regulation of the excitability of spinally projecting neurons in the PVN. We observed that SNAP and L-arginine both significantly increased the frequency of GABAergic mIPSCs of spinally projecting PVN neurons. Furthermore, the specific NO scavenger, carboxy-PTIO (Akaike et al. 1993), and an nNOS inhibitor, TRIM (Handy et al. 1995), completely blocked the effect of SNAP and L-arginine on mIPSCs. Thus the observed effect of SNAP and L-arginine on mIPSCs of PVN neurons is due to NO release and generation. Since the spontaneous mIPSCs recorded in the PVN slice preparation reflect the quantal release of GABA, these data suggest that NO increases the quantal GABA release and that the likely site of action of NO is the presynaptic GABAergic terminals. The strength of synaptic transmission can be altered through modulation of transmitter release probability and postsynaptic responsiveness. Analysis of frequency and amplitude/shape of mEPSCs and mIPSCs has been used to distinguish between pre- and postsynaptic loci of interventions (Kabashima et al. 1997; Ozaki et al. 2000; Sulzer and Pothos 2000). From the quantal hypothesis, only presynaptic actions can affect the probability of neurotransmitter release. Alterations in the peak amplitude/shape of mEPSCs and mIPSCs can be explained by changes in postsynaptic responsiveness. Our data are consistent with recent studies showing the presynaptic effect of NO in other CNS sites (Fowler et al. 1999; Ozaki et al. 2000). We found that carboxy-PTIO and TRIM alone had no effect on mIPSCs. Although these data imply that endogenous NO may not play a major role in regulation of the GABAergic input in this slice preparation, these results should not be taken as an implication that endogenous NO does not regulate GABA release in the PVN in vivo. This is because the present study was conducted using thin brain slices in which many neural and humoral influences are removed.

In contrast to its action on GABAergic mIPSCs, SNAP and L-arginine had no significant effect on the frequency of glutamatergic mEPSCs in spinally projecting PVN neurons. The similar differential effect of NO on mIPSCs and mEPSCs has been reported in supraoptic neurons in perfused hypothalamic slices (Ozaki et al. 2000). Both GABA and glutamate are considered to be the two major neurotransmitters in the PVN (Cui et al. 2001; Decavel and Van den Pol 1990; Hermes et al. 1996). However, the GABAergic afferent terminal provides the predominant synaptic input to PVN neurons (Decavel and Van den Pol 1990; Roland and Sawchenko 1993; Tasker and Dudek 1993). Thus the inhibitory GABAergic input may play a major role in regulation of the excitability of spinally projecting PVN neurons.

Based on the observation that NO potentiated the inhibitory
GABAergic synaptic input to spinally projecting PVN neurons without an evident effect on the glutamatergic synaptic input, we hypothesized that NO could inhibit the excitability of spinally projecting PVN neurons through an increased presynaptic GABA release. We found that a majority (80%) of the recorded spinally projecting PVN neurons in this study exhibited spontaneous activity. Previous recordings of spinally projecting PVN neurons in anesthetized rats have shown that most of the neurons are quiescent at rest (Bains and Ferguson 1995; Lovick and Coote 1988). One possibility for this difference is that in the intact animal, spinally projecting PVN neurons are tonically inhibited by an extrinsic input that is lost in the slice preparation. Also, the anesthetics used in those in vivo studies may have inhibited the excitability of PVN neurons. In the present study, bicuculline alone produced a significant increase in the firing activity in all the cells examined, indicating the

FIG. 7. Lack of effect of SNAP on miniature excitatory postsynaptic currents (mEPSCs) in DiI-labeled PVN neurons. A: representative tracings from a DiI-labeled neuron in the PVN showing spontaneous mEPSCs during control, application of SNAP (100 μM), and application of CNQX (20 μM). Note that 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) completely eliminated mEPSCs. B and C: cumulative plot analysis of mEPSCs of the same neuron showing the distribution of the inter-event interval (B) and amplitude (C) during control and application of 100 μM SNAP. Neither the inter-event interval nor the amplitude of mEPSCs was affected by SNAP. D: superimposed averages of 100 consecutive mEPSCs obtained during control and SNAP application. The decay phase of mEPSCs was best fitted with a single exponential function. The decay time constant was similar during control (τ = 2.52 ms) and SNAP application (τ = 2.54 ms). E and F: summary data showing the effect of 100 μM SNAP on the frequency (E) and the amplitude (F) of mEPSCs in 6 DiI-labeled PVN neurons. Data presented as means ± SE. (Wilcoxon signed-rank test).

FIG. 8. Inhibitory effect of SNAP on the firing activity of a DiI-labeled PVN neuron. A: histogram showing the effect of 100 μM SNAP on spontaneous discharge frequency of a DiI-labeled PVN neuron. B: raw tracings showing the spontaneous discharge activity of the same cell during control, application of SNAP, and washout in the same neuron. Note that SNAP inhibited the discharge activity of the neuron in a reversible manner.
presence of a tonic inhibition by the GABAergic synaptic input to spinally projecting PVN neurons. SNAP and L-arginine both significantly inhibited the spontaneous activity of spinally projecting PVN neurons. Although a previous study has shown that perfusion of the tissue slice with NO-containing aCSF elicits a small membrane depolarization of type II neurons in the PVN (Bains and Ferguson 1997a), the projection sites of PVN neurons were not determined in that study. In this study, we found that although SNAP and L-arginine slightly depolarized the spinally projecting PVN neurons, these NO-producing agents consistently inhibited the excitability of these neurons. Thus the predominant effect of NO is inhibition of spinally projecting PVN neurons likely through potentiation of presynaptic GABA release. Furthermore, SNAP and L-arginine failed to inhibit the spontaneous activity of PVN neurons in the presence of bicuculline. L-arg: L-arginine; Bic: bicuculline.

FIG. 9. Effects of L-arginine and bicuculline on the discharge activity of a DiI-labeled PVN neuron. A: histogram showing the effect of 100 µM L-arginine and L-arginine plus 20 µM bicuculline on the firing activity of a DiI-labeled PVN neuron. B: original tracings recorded during control, application of L-arginine, bicuculline alone, and L-arginine plus bicuculline from the same neuron shown in A. Note that L-arginine failed to inhibit the impulse activity of the neuron in the presence of bicuculline. L-arg: L-arginine; Bic: bicuculline.

In summary, this integrative study provides important new evidence for the mechanisms through which the activity of spinally projecting PVN neurons is regulated by NO. The NO-releasing agents, SNAP and L-arginine, both significantly increased the frequency of GABAergic mIPSCs but did not affect the glutamatergic mEPSCs of PVN neurons. Furthermore, SNAP and L-arginine significantly inhibited the excitability of spinally projecting PVN neurons, and such an effect was eliminated in the presence of bicuculline. Collectively, data from the present study provide strong evidence that NO inhibits the excitability of spinally projecting PVN neurons through potentiation of the GABAergic synaptic input. This new information is important for our understanding of the synaptic mechanisms involved in the regulation of spinally projecting PVN output neurons and their potential role in the autonomic control.

FIG. 10. Effects of SNAP, L-arginine, and bicuculline on the spontaneous activity of DiI-labeled PVN neurons. A: effects of SNAP, bicuculline, and SNAP plus bicuculline on the frequency of spontaneous activity of 6 DiI-labeled PVN neurons. B: effect of L-arginine, bicuculline, and L-arginine plus bicuculline on the frequency of spontaneous activity of 6 DiI-labeled PVN neurons. Bic: bicuculline. L-arg: L-arginine. *P < 0.05 compared to control; #P < 0.05 compared to recovery (Kruskal-Wallis test).
The authors thank R. Myers for technical support with the confocal microscope and P. Myers for secretarial assistance.

This study was supported by grants from the National Institutes of Health (HL-60026, GM-64830, and HL-04199). D. P. Li is currently supported by a postdoctoral fellowship award funded by the American Heart Association, Pennsylvania-Delaware Affiliate. H. L. Pan was a recipient of an independent scientist award supported by the National Heart, Lung, and Blood Institute during the course of this study.

REFERENCES


Bains JS and Ferguson AV. Nitric oxide regulates NMDA-driven GABAergic inputs to type I neurones of the paraventricular nucleus. J Physiol (Lond) 499: 733–746, 1997b.


