Effect of nitric oxide on excitatory amino acid-evoked discharge of neurons in NTS

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Submitted 17 January 2002; accepted in final form 22 August 2002

Dias, Ana Carolina Rodrigues, Eduardo Colombari, and Steven W. Mifflin. Effect of nitric oxide on excitatory amino acid-evoked discharge of neurons in NTS. Am J Physiol Heart Circ Physiol 284: H234–H240, 2003; 10.1152/ajpheart.00037.2002.—N-methyl-d-aspartate (NMDA) and non-NMDA excitatory amino acid (EAA) receptor subtypes are involved in the integration of visceral afferent inputs within the nucleus of the solitary tract (NTS). Microinjection studies indicate interactions between nitric oxide (NO) and EAA receptors within the NTS. To examine these interactions at the single cell level, this study characterized the effects of the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (l-NAME) and the NO donor 3-[2-hydroxy-2-nitroso-1-propylhydrazino]-1-propanamine (PAPA-NONOate) on the excitatory responses of vagus nerve (VN)-evoked NTS neurons to the activation of (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors in rats. Iontophoresis of l-NAME did not alter spontaneous or VN-evoked discharges, but significantly decreased the number of action potentials (APs) evoked by iontophoretic application of AMPA. The effects of l-NAME on NMDA-evoked discharge were variable; for the population, l-NAME did not change the number of APs evoked by NMDA. PAPA-NONOate enhanced the spontaneous discharge and the number of APs elicited by AMPA but not NMDA. Iontophoresis of the inactive enantiomers Nω-nitro-L-arginine methyl ester and hydroxydiazene sulfonic acid 1-oxide disodium salt had no effect on AMPA-evoked discharge. Our data suggest that NO facilitates AMPA-mediated neuronal transmission within the NTS.

nucleus of the solitary tract; N-methyl-d-aspartate; (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; vagus nerve stimulation

IN VIVO AND IN VITRO STUDIES report that the activation of glutamatergic receptors in the central nervous system increases the production and release of nitrosothiol substances (9, 29, 42). Nitric oxide (NO) is an unstable radical that undergoes either nitrosation chemistry or reaction with superoxide (14, 32, 39). The product of these reactions can affect modulatory sites on excitatory amino acid (EAA) receptors and alter neuronal responses to activation of these receptors (11, 17, 28, 32, 39, 40).

NO modulation of EAA responses can result in facilitation or inhibition depending on the specific brain area affected. NO facilitates responses to EAAs within the hippocampus (12, 13), the lateral geniculate nucleus (5), and the ventrobasal thalamus (37), to provide a few examples. In other areas, such as the cerebellum (38) and the periaqueductal grey (24), NO appears to mediate or facilitate inhibition. In certain areas, NO modulation of EAA receptors is even more complicated: in the dorsal horn of the spinal cord, inhibition of NO synthase (NOS) attenuates N-methyl-d-aspartate (NMDA) responses and facilitates (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) responses (2).

The first synapses within the central nervous system for the visceral afferent fibers of the vagus nerve (VN) occur in the nucleus of the solitary tract (NTS). Numerous studies have reported that these afferent terminals release L-glutamate and NO among other neurotransmitters (7, 10, 21). Vagal afferents to the NTS contain NOS (16, 21), and vagal deafferentation decreases NO immunoreactivity in the NTS (25). Immunohistochemical approaches have demonstrated the colocalization of neuronal NOS (nNOS) and the NMDA receptor subunit NMDAR1 as well as the AMPA receptor subunit GLuR1 in single neurons in the NTS of rats (22). NOS immunoreactivity has also been found in neurons in many nuclei within the medulla oblongata that send projections to the NTS, which suggests that NO generated in the NTS may modulate vagal afferent integration (7). Therefore, sources of NO within the NTS are peripheral afferent inputs to the nucleus, local interneurons within the NTS, and inputs from other central sites to the NTS.

Physiological studies also suggest a role for NO within the NTS in the modulation of cardiovascular function. Microdialysis and microinjection studies have shown that NOS inhibitors reduce extracellular levels of glutamate (19) and the cardiovascular responses to NTS microinjection of ionotropic EAA ago-
nists (20, 30). Activation of cardiopulmonary afferent inputs results in the release of NO, which activates soluble guanylate cyclase within the NTS (18). Micro-injection of soluble guanylate cyclase inhibitors into the NTS reduces the cardiovascular responses to microinjection of ionotropic EAA agonists into the NTS (42). Single unit recording studies from NTS neurons indicate that NOS inhibitors decrease spontaneous discharge (26) and NO donors increase spontaneous discharge.

All of these results suggest that NO may modulate visceral afferent integration within the NTS by modulating neuronal responses to EAA receptor activation. The specific aim of this study was to use single unit recording and iontophoretic techniques to examine the modulation by NO of NMDA- and AMPA-evoked discharges in VN-evoked NTS neurons.

MATERIALS AND METHODS

Animals. Experiments were performed on Sprague-Dawley rats (300–400 g body wt; Charles River). Rats were housed two per cage in a laboratory animal room and had unrestricted access to food and water. All animals were given at least 1 wk to acclimate to the environment before use. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Preparation. Animals were anesthetized with Inactin (100 mg/kg ip; Sigma) and placed on a thermostatically controlled heating pad. The trachea was cannulated and the rat was artificially ventilated with room air supplemented with 100% O2. The cervical VN including the aortic and laryngeal branches ipsilateral to the site of central recording was isolated and marked with a small loop of black suture. Catheters were placed in the femoral artery and vein for blood pressure monitoring and injection of drugs. Arterial pressure was measured using a Cobe CDX transducer (Cobe Laboratories; Lakewood, CO). Mean arterial pressure (in mmHg) and heart rate (in beats/min) were determined from the pulsatile signal using a Coulbourn blood pressure processor. Gallamine thiethiodide (20 mg/kg initially supplemented with 5 mg as needed, typically every hour) was also given for paralysis. Depth of anesthesia was gauged by the stability of blood pressure and heart rate and the absence of a pressor response to paw pinch. The rat was placed in a stereotaxic frame, and an occipital craniotomy was performed to expose the dorsal surface of the medulla in the region of obex. The previously isolated VN was placed on bipolar stimulating electrodes and covered with a mixture of mineral oil and Vaseline petroleum jelly.

Electrophysiological recordings. Extracellular action potential (AP) discharge was recorded with a five-barreled electrode. The recording barrel was filled with a solution of 0.5 M sodium acetate that contained 2% Chicago sky blue. One barrel of each five-barreled electrode was filled with a solution of 3 M NaCl and used for current balancing and pH controls. The other barrels were filled with different drug solutions (see Drugs and drug administration). Recordings were obtained from the region 1.2 mm caudal and 0.5 mm rostral to the calamus scriptorius, 0–0.8 mm lateral to the midline, and 0.2–1 mm below the surface. The electrode was lowered into the tissue in 2.0–μm steps by a step-driver controller (Burleigh Instruments; Fisher, NY). The VN was stimulated with single pulses of 1 ms in duration, 0.5 Hz, and 500 μA. If the neuron responded with an AP to each of two VN stimuli separated by 5 ms, the input was considered monosynaptic; otherwise, the input was considered polysynaptic.

APs were recorded and amplified by a direct current amplifier (World Precision Instruments; New Haven, CT) passed through an alternating current filter and sent to a digital oscilloscope (Nicolet Instruments; Madison, WI), an audio monitor (Grass Instruments; Quincy, MA), a videotape recorder (A. R. Vetel; Redburg, PA), and a window discriminator (World Precision Instruments; Sarasota, FL). The window-discriminator output was led to a Cambridge Electronic Design (model CED1401; Cambridge, UK) analog-to-digital converter interfaced with a personal computer. For on-line and off-line analyses, Spike 2 data-acquisition software was used.

Drugs and drug administration. Drugs were administered by application of microiontophoretic ejection currents to the drug-containing barrels. The drug solutions for microiontophoresis were 10 mM AMPA (Tocris Cookson; Bristol, UK), 100 mM NMDA (Sigma; St. Louis, MO), 50 mM N-g-nitro-L-arginine methyl ester (l-NAME, NOS inhibitor; Sigma), 50 mM N-g-nitro-o-arginine methyl ester (o-NAME; Sigma), 50 mM 1-(3-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA-NONOate or NOC-15, a NO donor; Sigma), and 50 mM hydroxysapinsulic acid 1-oxide disodium salt (sulfo-NONOate, a negative control of PAPA-NONOate; Sigma). Drugs were dissolved in 150 mM NaCl, and pH was adjusted to 8.0–8.5 for AMPA and NMDA, 6.5 for l-NAME and o-NAME, and 8.0 for PAPA-NONOate and sulfo-NONOate. All drugs were ejected as anions. Retaining currents of appropriate polarity were applied to the drug barrels to retain the passive diffusion of the drug from the electrode tip during nonejection periods.

NO modulation of EAA responses was studied as follows: after the baseline spontaneous discharge rate was recorded, VN-evoked neurons were excited by pulsatile iontophoretic application of 10–20 nA of NMDA or AMPA (control) until a steady-state level of evoked discharge was reached. NMDA and AMPA were ejected for successive 7- to 8-s periods separated by 10- to 15-s intervals (Fig. 1). At this point, the iontophoresis of the excitatory agent was stopped, and a continuous iontophoretic ejection of l-NAME, PAPA-NONOate, or the respective enantiomer was begun. After ~2 min, the pulsatile application of AMPA or NMDA was begun again. After the AMPA- or NMDA-evoked discharge reached a steady state, iontophoresis of the excitatory agent and either the l-NAME, PAPA-NONOate, or the respective enantiomer was stopped. After a 3- to 5-min recovery period, the pulsatile application of AMPA or NMDA was begun again to determine whether altered responses had returned to control levels. To examine NO modulation of synaptic inputs, poststimulus time histograms of 30 responses to 0.5-Hz VN stimulation were obtained before, during, and after iontophoresis of l-NAME.

Data analysis. Neuronal responses to iontophoretic application of NMDA and AMPA were obtained by subtracting the number of APs during the drug ejection period from the number of APs measured during an equivalent-duration baseline period. The number of APs was counted during a 10-s period that began with the onset of the iontophoretic ejection pulse. AMPA- and NMDA-evoked discharges were averaged over three or four cycles once the level of discharge had reached the steady state. AMPA- and NMDA-evoked discharges are presented as absolute numbers of APs. Rate-meter records (no. of APs/s) were used for statistical analysis of the spontaneous discharge rate, and these data are presented as a frequency (in Hz). All data were analyzed by repeated-measures ANOVA and subsequent Student’s mod-
ified t-test with Student-Newman-Keuls test. Significance was accepted at a 0.05 confidence level.

RESULTS

General characteristics of neurons studied. Successful recordings were obtained from 42 VN-evoked neurons in 26 rats. The average onset latency for VN-evoked excitation was 2.5 ± 0.2 ms. In 8 neurons, the vagal afferent input exhibited characteristics consistent with a monosynaptic input (onset latency, 2.3 ± 0.5 ms), whereas in the remaining 34 neurons, the input appeared to be polysynaptic (onset latency, 2.6 ± 2.2 ms). There were no differences between these two groups in any of the parameters examined, and therefore they are grouped together in the subsequent analyses. Spontaneous discharge was present in 34 neurons and averaged 1.1 ± 0.2 Hz.

Responses for 17 of the 34 neurons were observed after injection of 16 μg of phenylbiguanide into the right atrium to discern a thoracic cardiopulmonary site of origin for the vagal afferent input. Of the 17 neurons, 10 were excited, 5 were inhibited, 1 did not respond, and 1 responded with an excitatory response followed by an inhibitory response.

Effects of L-NAME on AMPA- and NMDA-evoked discharges in VN-evoked NTS neurons. Iontophoresis of L-NAME (20–80 nA, 2–5 min) did not alter spontaneous discharge rates (2.4 ± 0.5 vs. 2.5 ± 0.7 Hz; n = 15). Iontophoresis of L-NAME had no effect on discharge evoked by VN stimulation (no. of APs evoked by 30 stimuli at 0.5 Hz: control, 26 ± 3; during iontophoresis of L-NAME, 27 ± 3; n = 11).

The number of APs evoked by pulsatile AMPA iontophoresis was reduced during coiontophoresis of L-NAME (Fig. 1). For the population, AMPA-evoked discharge was decreased from 44 ± 7 to 29 ± 6 APs (P = 0.002; n = 14; Fig. 2). As a control for possible nonspes-
specific effects of L-NAME, the inactive enantiomer D-NAME was applied in a manner similar to that described for L-NAME. Iontophoresis of D-NAME (40 nA) did not alter spontaneous discharge (control, 3.0 ± 0.5; D-NAME, 2.8 ± 0.5 Hz; n = 5). In addition, D-NAME did not alter the number of APs evoked by AMPA (control, 64 ± 12; D-NAME, 69 ± 9; n = 5).

L-NAME did not significantly alter the number of APs evoked by pulsatile application of NMDA (see Fig. 1). Under control conditions, NMDA iontophoresis evoked an average of 50 ± 10 APs, and, during iontophoresis of L-NAME, NMDA evoked 45 ± 9 APs (n = 15; Fig. 2).

Although the effects of L-NAME on NMDA-evoked discharge were not significant for the population, the iontophoresis of L-NAME caused variable effects on neurons within the population. The number of NMDA-evoked APs was reduced by 12–60% during iontophoresis of L-NAME in 6 cells. In 7 cells, NMDA-evoked discharge changed by only ±10% during L-NAME iontophoresis, which was within the normal range of variability. In 2 cells, L-NAME enhanced NMDA-evoked discharge by 23 and 50%.

Given the variable effects of L-NAME on NMDA-evoked discharge, the question arises as to whether NMDA- and AMPA-evoked discharges were altered in a similar or dissimilar manner by L-NAME. There was no relationship between the L-NAME effect on NMDA-evoked discharge and the L-NAME inhibition of AMPA-evoked discharge for individual neurons (Fig. 3). In all neurons, L-NAME decreased responses to AMPA receptor activation regardless of the effect on NMDA-evoked discharge.

In one neuron, the recording was unusually stable, and it was possible to examine the effects of L-NAME over a much broader range of AMPA- and NMDA-evoked discharges (Fig. 4). Increasing the AMPA or NMDA iontophoretic ejection current while keeping duration constant resulted in a dose-related increase in the number of evoked APs. In this cell, iontophoresis of L-NAME had no effect on AMPA- or NMDA-evoked discharges until the EAA-evoked discharge exceeded 150 APs in the 10-s measurement period.

Attempts to overcome L-NAME inhibition with L-arginine were unsuccessful, because L-arginine appeared to have nonspecific effects on neuronal discharge. Iontophoresis of L-arginine decreased spontaneous discharge and AMPA-evoked discharge rates in seven of nine neurons tested with no recovery to the control level of AMPA-evoked discharge observed after cessation of the L-arginine iontophoresis.

Effects of PAPA-NONOate on AMPA- and NMDA-evoked discharges of VN-evoked NTS neurons. In 12 VN-evoked neurons, AMPA-evoked discharge was examined during iontophoresis of the NO donor PAPA-NONOate using the same protocol as in the L-NAME studies. Two cells had no spontaneous discharge before and during iontophoresis of PAPA-NONOate, and one cell had an inordinately high spontaneous discharge rate of 8.8 Hz and was excluded from analysis. The NO donor increased the spontaneous discharge of six of the remaining nine cells from a control level of 1.8 ± 3 to 3.2 ± 7 Hz during iontophoresis of PAPA-NONOate (P = 0.03; n = 9; Fig. 5A).
The number of APs evoked by pulsatile iontophoresis of AMPA was significantly enhanced during iontophoresis of the NO donor (control, 51 ± 10; during PAPA-NONOate, 82 ± 16; \( P = 0.04; n = 12 \); Fig. 5B). Iontophoresis of PAPA-NONOate did not significantly alter NMDA-evoked discharge (control, 47 ± 8; during PAPA-NONOate iontophoresis, 51 ± 10; \( n = 5 \)).

As a control for nonspecific effects of PAPA-NONOate, the effects of the inactive enantiomer sulfo-NONOate were examined. Sulfo-NONOate did not alter the rate of spontaneous discharge (control, 0.8 ± 0.4; sulfo-NONOate, 0.7 ± 0.4 Hz) or the number of APs evoked by AMPA application (control, 46 ± 14; during sulfo-NONOate iontophoresis, 48 ± 14; \( n = 6 \)).

DISCUSSION

This study shows that AMPA receptor-mediated excitation of NTS neurons that receive vagal afferent inputs can be facilitated by NO. Inhibition of NOS by \( L \)-NAME significantly decreased the number of APs evoked by AMPA receptor stimulation. The NO donor PAPA-NONOate significantly enhanced the rate of spontaneous discharge and the number of APs evoked by AMPA. The inhibition of NOS by \( L \)-NAME did not significantly decrease the number of APs evoked by NMDA receptor stimulation when measured for the population as a whole. In the majority of NTS neurons, NMDA-evoked discharge was not altered by inhibition of NOS. However, there was a small number of neurons in which NMDA-evoked discharge was either facilitated or attenuated during iontophoresis of \( L \)-NAME. Reciprocal effects of NO on AMPA- vs. NMDA-evoked discharge have been reported for the spinal cord dorsal horn (2).

In the present study, iontophoresis of \( L \)-NAME decreased the number of APs evoked by AMPA receptor stimulation in all neurons tested, but the number of APs evoked by NMDA receptor activation was decreased in 40% of the cells. This result was surprising, because the relationship between NO and NMDA receptors has been studied in other systems, where it has been shown that NMDA receptor activation results in an entrance of \( \text{Ca}^{2+} \) into the cell that activates NOS and reduces subsequent NMDA-evoked responses (11, 17, 27). Such an inhibitory feedback mechanism operating during prolonged activation of NMDA receptors could be a protective mechanism against glutamate-induced neurotoxicity (39). It is possible that the timing of administration or the concentration of NO donors and NOS inhibitors during iontophoretic application might explain why we observed variable changes in NMDA-evoked discharge during the \( L \)-NAME treatment. Sensitivity to NO may vary in different populations of cells (39). Therefore, interactions between NMDA receptors and NO in the NTS might be related to the specific reflex function(s) of a given cell.

The role of ionotropic glutamate receptors (NMDA and non-NMDA) in the integration of visceral afferent inputs within the NTS has been well demonstrated (1, 3, 8, 15, 33, 35, 36, 44). Many observations also suggest a role for NO within the NTS in cardiovascular regulation. Intravenous administration of \( L \)-NAME reduced the vasodilation in the hindquarter bed caused by glutamate microinjection into the NTS (4), which sugg-
gests modulation of glutamatergic excitation by nitro-dergic mechanisms within the NTS. Other in vivo studies showed that microinjection of l-arginine reduced heart rate, renal sympathetic nerve activity (RSNA), and blood pressure, whereas microinjection of a NOS inhibitor increased RSNA and blood pressure (43). These effects may be dependent on ionotropic glutamate receptors, because the microinjection of NMDF and non-NMDA receptor antagonists decreased the effects of l-arginine (20).

Visceral afferent inputs to the NTS can generate NO, and this NO may modulate afferent integration in the dorsal vagal complex and autonomic reflex function (18, 34). Vagal afferents and terminals that contain nNOS are present in the NTS, and deafferentation of VN fibers causes a reduction in NOS staining in the NTS (16, 23, 34, 42). The differential distribution of nNOS in the NTS supports the idea of NO modulation of autonomic reflexes (22). The presence of NOS immunoreactivity colocalized with NMDA receptor subunit NMDAR1 and AMPA receptor subunit GluR1 in the NTS of the rat suggests a modulatory role of NOS activity on EAA receptor activation (43). The subunits NMDAR1 and GluR1 are mainly localized on postsynaptic structures, and our study observed the postsynaptic discharge evoked by stimulation of these receptors on NTS neurons.

Our studies cannot definitively determine whether NO modulates EAA-evoked responses at presynaptic and/or postsynaptic sites. Our results suggest that this modulation can occur at a postsynaptic site, because our protocol examined effects of direct application of EAA agonists independently of synaptic inputs. However, NO-stimulated release of glutamate might contribute to the enhanced spontaneous discharge rate observed during iontophoresis of the NO donor in our study. The administration of NO via microdialysis induced glutamate release, whereas NOS inhibition reduced the basal level of glutamate in the NTS (19). A recent study demonstrated a facilitatory effect of NO on the release of glutamate when NMDA receptors were stimulated in the NTS, which enhanced the hypertension and bradycardia that is normally observed during activation of NMDA receptors in NTS (30). Therefore, although our data suggest a postsynaptic site for NO-EAA interactions, it does not preclude presynaptic interactions.

The lack of inhibition of VN-evoked discharge during iontophoresis of l-NAME was surprising to us, as we predicted that NO would modulate synaptic inputs. However, the lack of inhibition is likely the result of our protocol. We examined VN-evoked discharge using a low stimulus frequency, 0.5 Hz. We speculate on the basis of data obtained from the cell illustrated in Fig. 4 that NO may be of functional importance during periods of high-frequency discharge, and our VN stimulus paradigm did not evoke high-frequency discharge. Similarly, we failed to observe a reduction in spontaneous discharge rate during iontophoresis of the NOS inhibitor, whereas two previous studies of NTS neurons have shown such a reduction (26, 41). This difference could be explained by the relative discharge frequen-

cies of the NTS neurons. In the present study, spontaneous discharge was low (1–3 Hz), whereas in the study of Ma et al. (26), spontaneous discharge frequency was 5–6 Hz. Absolute frequencies were not reported in the study of Tagawa et al. (41). The increase in spontaneous discharge that was observed during iontophoresis of the NO donor could be the result of modulation of synaptic inputs; however, it could also be the result of changes in postsynaptic excitability.

The decrease in the number of APs evoked by AMPA during NOS inhibition suggests that NO facilitates AMPA transmission within the NTS. Consistent with this, we observed a significant increase in the number of APs evoked by AMPA microiontophoresis in the presence of the NO donor PAPA-NONOate. This compound is considered to be a pure NO donor in that it releases only NO (40) and does not release other substances that could interfere with the neuron or affect NOS activity. Our data reinforce the hypothesis that AMPA receptor-evoked excitation can be facilitated by NO. The lack of effect on low-frequency VN-evoked discharges and the dose-response relationship obtained from one neuron suggest that this facilitation may only come into play when the neuron is driven into a high-discharge-frequency state. This would provide a means of sustaining excitatory transmission rate during periods when transmitter depletion or other factors might otherwise reduce excitation.

The authors gratefully acknowledge the support of National Heart, Lung, and Blood Institute Grants HL-41894 and HL-56377 (to S. W. Mifflin). A. C. R. Dias was partially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Award BEX 076/00-1.

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