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Review

Nitric oxide as a modulator of central respiratory rhythm in the isolated brainstem of the bullfrog (*Rana catesbeiana*)☆

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Abstract

Nitric oxide (NO) is a unique interneuronal neurotransmitter and/or neuromodulator that is involved in a variety of physiological functions within the central nervous system (CNS). In neural tissue, NO is generated from an oxygen-dependent, constitutive NO synthase (NOS) by glutamatergic stimulation of *N*-methyl-D-aspartate (NMDA) receptors. Recent studies indicate that NO has excitatory effects on breathing within the CNS and mediates a central component of the hypoxic ventilatory reflex in mammals. Because NMDA receptors are important in central respiratory rhythmogenesis, we hypothesized that NO would have significant effects on the central pattern generator (CPG) for breathing in the brainstem. To test this hypothesis, the effects of NO on respiratory-related neural activity were investigated using an in vitro brainstem preparation from North American bullfrogs (*Rana catesbeiana*). Extracellular recordings of respiratory-related burst activity were made from cranial nerves V, X and XII before and during superfusion of the brainstem with NO-generating compounds, or inhibitors of NO synthesis. Addition of the NO donor, sodium nitroprusside (SNP; 0.1–1.0 mM), or the amino acid precursor for NO synthesis, L-arginine (L-Arg; 0.01–1.0 mM), caused significant increases in respiratory-related burst frequency. Inhibition of NOS with N^{ω}-nitro-L-arginine (L-NA; 5–10 mM), a non-selective NOS inhibitor, caused a significant reduction in burst frequency or reversibly abolished neural activity. Brainstem perfusion with the specific neuronal NOS (nNOS) inhibitor, 7-nitro indazole (7-NI), produced significant, dose-dependent reversible reductions in burst frequency at concentrations of 0.1, 0.5 and 1.0 mM. These results suggest that production of NO, probably via nNOS, provides an excitatory input to the respiratory CPG in the amphibian brainstem. Our results suggest that NO may be a necessary inter- or intracellular messenger for neurotransmission and/or neuromodulation of central respiratory drive to motor effectors in the bullfrog. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Nitric oxide; Breathing; Central pattern generator; Amphibian; L-Arginine; 7-Nitro indazole; Sodium nitroprusside; nNOS

1. Introduction

Nitric oxide (NO) is a unique, ubiquitous chemical messenger that is involved in a diverse array of physiological functions in the central and peripheral nervous systems of vertebrates and invertebrates (see Ref. [12] for review). In neurons, NO is synthesized by glutamatergic stimulation of *N*-methyl-D-aspartate (NMDA) receptors which activate a calcium-dependent neuronal NO synthase (nNOS) in the post-synaptic cell [4,5]. Because NO is freely diffusible across cell membranes, it is not spatially restricted to the conventional anterograde synaptic pathway as are 'classical' neurotransmitters [4,35]; therefore, NO may act both as an intracellular and intercellular messenger molecule. These unique properties suggest that, where NO is produced in the nervous system, there is significant potential for interaction between NO and a large number of nearby synapses [21]. In this context, it has been suggested that the diffusible nature of NO may be important for synchronizing neural networks that perform specific neural tasks, or provide a neural substrate

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for motor behaviors [1,25,27,28]. Thus, non-synaptic communication by NO may play an important role in the modulation of networks involved in central respiratory rhythm. Although direct evidence for non-synaptic modulation of neural network function is lacking in vertebrates, recent evidence suggests that NO may act as an excitatory non-synaptic neurotransmitter between feeding neurons within the buccal ganglia of the gastropod mollusc, *Lymnea stagnalis* [29].

Respiratory rhythm is generated from an unidentified central neural network oscillator located in the rostral region of the brainstem in vertebrates [2,6,11]. In mammals, recent experiments have shown that the pre-Bötzinger complex (pre-BötC), located in the rostral ventrolateral medulla, may be both necessary and sufficient for respiratory rhythm generation [31]. Neurons with bursting properties have been located in the pre-BötC, and these bursting neurons are hypothesized to provide the source of respiratory rhythm generation [31,34]. However, the 'shaping' of the ongoing respiratory rhythm to provide the precise spatiotemporal motor output to respiratory muscles (i.e. pattern generation) is clearly a property of synaptic network interactions [31,34]. Because bursting neurons are embedded within a neural network, it has been difficult to separate respiratory rhythm generation from pattern formation [31]. In general, regardless of the precise mechanism(s) used to generate respiratory rhythm and pattern, the term 'central pattern generator' (CPG) is often used to denote a neural network that drives a particular motor behavior and may or may not involve separate rhythm and pattern-forming neural circuits [23].

Breathing is modified by a large number of excitatory and inhibitory neurotransmitters and neuromodulators within the CNS [3], and studies from mammals indicate that glutamatergic stimulation of non-NMDA and NMDA receptors is necessary for generation and neurotransmission of respiratory rhythm, and for respiratory timing [7,31,32,34]. NO has been shown to play an important role in a pontine phase-switching function [22] and is important in mediating central hypoxic ventilatory reflexes [15,16]. In amphibians, microinjection of glutamate into rostral brainstem areas stimulates fictive breathing [24]; however, it is unknown what specific glutamate receptors mediate this response. At present, there is little information on the potential role of NO in the central regulation of breathing despite the known link between NMDA receptors, neuronal NO synthesis and breathing.

Modulation of the respiratory CPG by NO in non-mammalian vertebrates has not been examined. In a previous study from this laboratory, the effects of nitrergic drugs on respiratory frequency in the isolated bullfrog brainstem preparation were reported [17]. The results from that study indicated that NO stimulates respiratory frequency; however, other components of respiratory CPG modulation such as respiratory burst and pattern formation were not reported. The purpose of the present study was to determine whether NO plays a role in modulating the vertebrate respiratory CPG using an in vitro brainstem preparation from the North American bullfrog (*Rana catesbeiana*).

2. Materials and methods

Twenty-six bullfrogs (100–190 g) that were used for a previous study [17] were used in this study. Frogs were purchased from a commercial supplier (West Jersey Bio. Services, Wenonah, NJ) and maintained in a semi-aquatic environment at room temperature (22–24°C). For surgery, the animals were initially anesthetized with a 1% solution of tricaine methanesulfonate (MS 222; Sigma) buffered to pH 7.4 until breathing movements ceased (ca 10–15 min). The animal was then removed and placed in crushed ice for 1 h to reduce metabolism for subsequent dissection of brain tissue and to ensure maintenance of anesthesia. After removal from the ice, areflexia was established and a small opening was made in the cranium using a dental drill. The forebrain rostral to the optic lobes was transected and removed by suction. During decerebration and subsequent dissection, the brainstem was continuously perfused with cold (5–7°C), oxygenated artificial CSF (aCSF) with a composition used in a previous study with this species [18]. The brainstem was transferred to a recording chamber (7 ml) and superfused with aCSF at 5–10 ml/min from a reservoir maintained at room temperature (21–23°C) and bubbled with oxygenated, isocapnic (98% O_2/2% CO_2) aCSF. The brainstem was pinned ventral side up in a recording chamber lined with sylgard (Dow Corning) and the dura removed. Extracellular recordings were obtained with suction electrodes attached to the rootlets of cranial nerves V (trigeminal), X (vagus) and XII (hypoglossal). These nerves innervate buccal elevators (trigeminal and hypoglossal) and depressors (hypoglossal) in the oropharyngeal region of anurans and are responsible for generating airflow associated with small amplitude, non-ventilatory buccal oscillations and larger amplitude, positive-pressure lung ventilatory events [19]. The vagus nerve innervates the glottal musculature which controls airflow associated with lung ventilation. In the frog brainstem preparation in vitro, these cranial nerves exhibit respiratory-related neural bursts associated with buccal oscillation and lung ventilation observed in vivo [19]. Neural activity was amplified

10 000 times with a differential AC amplifier (A-M systems model 1700; Everett, WA), filtered (low pass, 100 Hz; high pass, 5 kHz) and recorded on tape (A.R. Vetter, model 402, Rebersburg, PA) for subsequent analysis; data were replayed from the tape recorder onto a computer (Power Macintosh, 7200) which interfaced with a data acquisition system sampling at 2 kHz (Maclab 8S; AD Instruments, Milford, MA).

An external perfusion reservoir, identical to and in parallel with the reservoir containing aCSF, was used to add drugs to the recording bath. Drugs were dissolved in aCSF and titrated to pH 7.4–7.6 prior to addition to the perfusion reservoir and bubbled with an oxygenated, isocapnic gas mixture. 7-NI was dissolved in 100% dimethylsulfoxide (DMSO) and diluted with aCSF to achieve the desired concentration of 7-NI in the perfusate (final concentration of 0.1–1.0% DMSO in aCSF). For the experiment, each brainstem was perfused with aCSF and switched to the test solution containing an individual drug in the external circuit for 20 min. Control perfusions were done by perfusing the brainstem with aCSF (or 1% DMSO for 7-NI) from the external circuit. In 19 preparations, SNP (0.1–1.0 mM; $n = 14$), L-Arg (0.01–1.0 mM; $n = 13$) or L-NA (5–10 mM; $n = 15$) were used to examine the effects of exogenous NO, NO production and NOS blockade, respectively, on respiratory-related burst activity. In these experiments, the respiratory-related neural responses to the range of concentrations were pooled, and no dose-dependent effects of these drugs were examined. In seven additional preparations, 7-NI was used to examine the effects of blockade of nNOS on respiratory-related neural activity. The dose-dependent effects of nNOS blockade was examined by perfusing each preparation with 0.1, 0.5 and 1.0 mM 7-NI.

The minimum concentration of each drug that affected neural burst activity in the bullfrog brainstem preparation was found in preliminary experiments. The concentrations of drugs used are in the range found to affect neural activity in brainstem slices containing the nucleus tractus solitarius (NTS) in rats [38]. All drugs were obtained from Sigma (St Louis, MO). For each preparation, the protocol consisted of perfusion with aCSF and switching to an identical reservoir containing a test (drug) solution for 20 min and then switching back to the original aCSF. The next drug solution or next concentration was given after respiratory activity had returned close to its original pre-drug activity. Thus, the respiratory activity during perfusion with each drug solution was compared with its own activity immediately prior to switching to the test solution. Significant differences in respiratory burst frequency, duration and amplitude between control and test solutions were determined by paired t-test. Significance was taken as $P < 0.05$.

3. Results

The bullfrog brainstem preparation exhibits a variety of respiratory-related motor behaviors in vitro including high frequency, low amplitude buccal activity, low frequency, high amplitude lung breaths, and episodic lung bursts (Fig. 1). These respiratory patterns have been well characterized in vivo [19,20] and the neural bursts associated with these patterns characterized using in vitro brainstem preparations [18,24,33]. In our study, each preparation produced a unique respiratory-related output with some preparations exhibiting a wide variety of burst patterns (Fig. 1A).

Fig. 2 summarizes the effects of SNP, L-Arg and L-NA application on burst frequency that we found in a previous study [17]. Perfusion of the brainstem with SNP significantly increased frequency by 70–80% within 10 min of perfusion and was sustained over the 20-min perfusion period ($t_{13} = 4.2$; $P < 0.002$; Fig. 2). The effect was entirely reversible in all preparations. L-Arg perfusion resulted in a 65% increase in burst frequency ($t_{12} = 2.41$; $P < 0.05$; Fig. 2), but there was more variability associated with L-Arg perfusion including an attenuation of burst frequency in four of 13 preparations. The general, competitive NOS inhibitor L-NA had no significant effect on burst frequency at concentrations below 5 mM; however, at higher concentrations (5–10 mM), frequency was significantly attenuated ($t_{14} = 5.58$; $P < 0.001$; Fig. 2); at these concentrations neural activity was reversibly abolished in 10 of 15 preparations.

The selective nNOS inhibitor, 7-NI, reversibly inhibited neural bursts in a dose-dependent manner from 0.1 to 1 mM (Fig. 3; Table 1) and reversibly abolished neural activity in all seven preparations at 1 mM (Table 1).

Although control perfusions with 1% DMSO did not significantly affect burst frequency, there were some changes in burst characteristics with DMSO in aCSF. Figs. 4 and 5 illustrate the effects of DMSO in two different preparations. In one preparation, DMSO perfusion created longer duration, higher amplitude 'complex bursts' with no overall change in burst frequency (Fig. 4). In a second preparation, there was a slight increase in burst amplitude with no change in burst duration. In this preparation, DMSO alone did not affect burst frequency, but did appear to have a stimulatory effect on fictive buccal oscillations (Fig. 5C). The overall effects of DMSO were not consistent in every preparation. DMSO increased burst duration from 2.2 ± 0.4 to 4.3 ± 1.3 s, but this

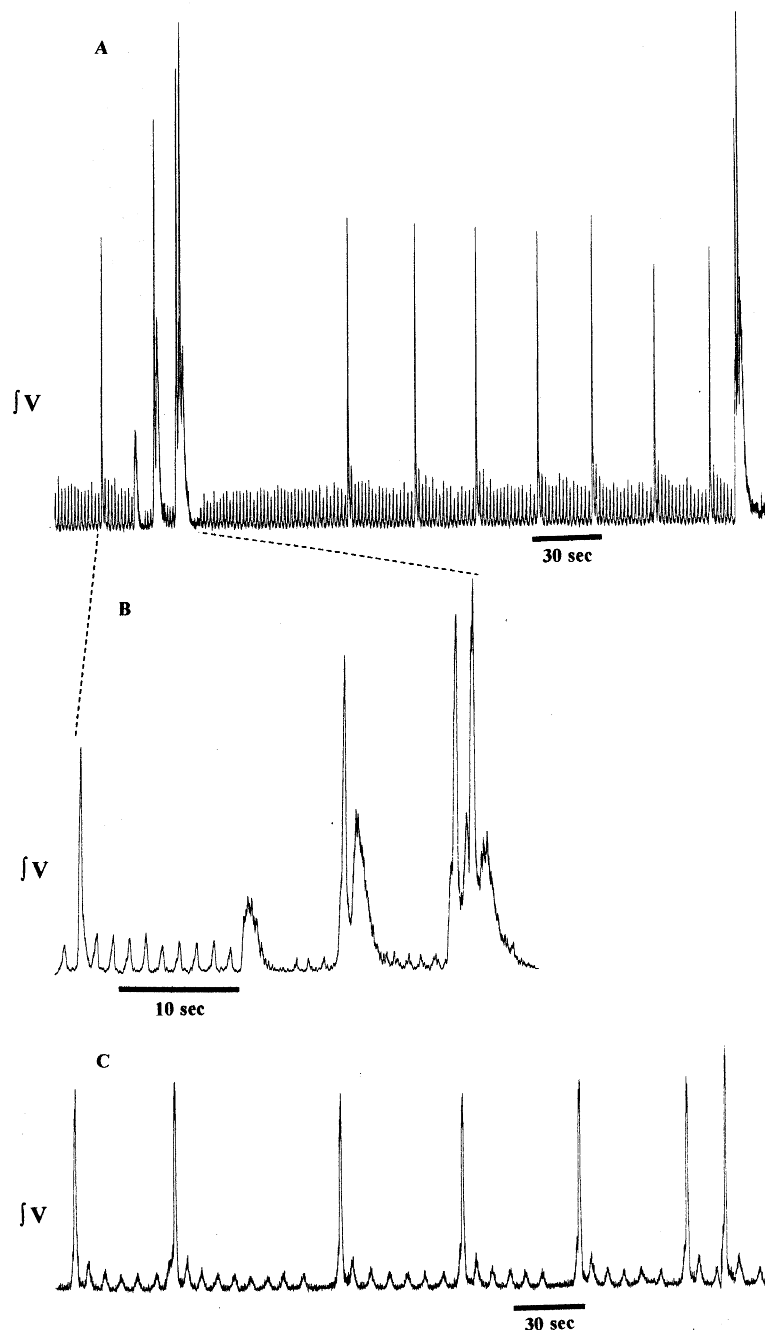

Fig. 1. Integrated trigeminal nerve activity (∫V) from two preparations perfused with normal (98% O_2/2% CO_2) aCSF showing variability of 'control' respiratory patterns. (A) Preparation exhibiting low amplitude, high frequency buccal oscillations, single lung bursts and 'episodic' lung bursts. (B) Increased sweep speed from a section of (A) illustrating each different type of respiratory-related burst in this preparation. (C) Preparation exhibiting a slower rate of buccal oscillations and lung bursts that occur singly with no episodic bursts.

change was not significant (Fig. 6). In addition, burst amplitude increased by an average of 50%, but this increase was also not significant (Fig. 6). In some cases DMSO appears to have a general stimulatory effect on pattern formation, burst duration and amplitude. The addition of 7-NI with DMSO appears to attenuate the stimulatory effects since the addition of 7-NI at 0.1 and 0.5 mM in DMSO reduced burst duration and amplitude to control (aCSF) values (Fig. 6).

4. Discussion

Conscious or decerebrate, paralyzed bullfrogs typically display three distinct types of ventilatory behaviors: high frequency, low amplitude buccal oscillations; low frequency, high amplitude lung breaths that occur singly or in episodes; and lung inflation cycles [19,20]. Several studies, including this study, using in vitro preparations from anuran amphibians have demon-

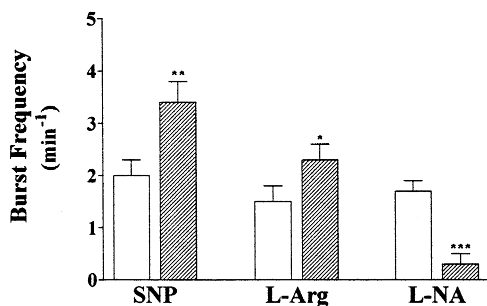

Fig. 2. Summary of respiratory related burst frequency (bursts min^{-1}) in response to brainstem perfusion with nitrergic drugs. Control (aCSF perfusion) rates (open bars) are compared with frequency during the 10–20 min perfusion with NO donors (SNP, sodium nitroprusside and L-Arg, L-arginine) or an inhibitor of NO synthesis (L-NA, nitro-L-arginine). *$P<0.05$; **$P<0.01$; ***$P<0.001$ from control (paired t-test). Adapted from Ref. [17].

Table 1
Effects of 7-nitroindazole (mM) and 1% DMSO (vehicle) on respiratory-related lung burst frequency (bursts min^{-1}) in *R. catesbeiana* brainstem preparations[a]

Condition	0.1 mM	0.5 mM	1.0 mM	DMSO
Control (aCSF)	1.1 ± 0.23	1.0 ± 0.13	1.2 ± 0.17	1.5 ± 0.34
Drug (7-NI or DMSO)	0.70 ± 0.14	0.40 ± 0.14*	0**	1.2 ± 0.30
Recovery (aCSF)	1.0 ± 0.17	1.0 ± 0.22	0.9 ± 0.27	0.9 ± 0.22

[a] Values are mean ± S.E.M. ($N=7$). Significance determined by paired t-test between control (aCSF) and 7-NI (0.1, 0.5 and 1.0 mM) or DMSO.
* $P<0.02$.
** $P<0.001$.

strated respiratory-related neural bursts that correspond to the ventilatory behaviors characterized in vivo [18,24,33]. Thus, the in vitro brainstem preparation has proven useful for examining the mechanisms for respiratory rhythm generation in amphibians [30] and other vertebrates. A recent study suggests that this preparation may have limitations for studying respiratory pattern formation because a wide repertoire of motor patterns are produced that may not be respiratory-related [33]. In our study, we have also noted a variety of burst patterns among different preparations and it is difficult to determine whether each burst is indeed respiratory-related (Fig. 1). However, the primary aim of our study was to assess whether respiratory-related activity is affected by modulators of nitric oxide (NO) and we have examined neural activity before, during and after perfusion of a particular neuromodulator of NO. Thus, although each preparation may be unique in terms of a particular motor output, our protocol allows us to examine the specific effects of neuromodulators in each preparation. In these experiments, we have not observed any specific changes in respiratory pattern (e.g. single breaths to episodic breathing) after modulation of NO. It is possible that some of the variation in respiratory-related motor output between preparations may be related to residual effects of MS-222 anesthesia and/or reduced metabolism. However, our baseline respiratory activity in vitro with *R. catesbeiana* preparations is very close to other studies where MS-222 anesthesia was not used [18,24], suggesting that anesthesia alone cannot account for the variability in fictive breathing patterns in this species.

Previous experiments with the bullfrog brainstem preparation have shown that microinjection of glutamate into rostral areas of the medullary reticular formation near the facial motor nucleus significantly increases respiratory burst frequency, whereas microinjections at more caudal locations inhibits neural output or has no effect [24]. That study did not identify specific receptors responsible for mediating the effects of glutamate; however, it is well established that glutamate released from CNS neurons, acting at NMDA receptors, produces NO by a calcium/calmodulin-dependent cyclic GMP (cGMP) pathway in neurons [4,5]. In the frog brainstem preparation, the link between NMDA receptor stimulation and NO production has not been conclusively established, but our results are consistent

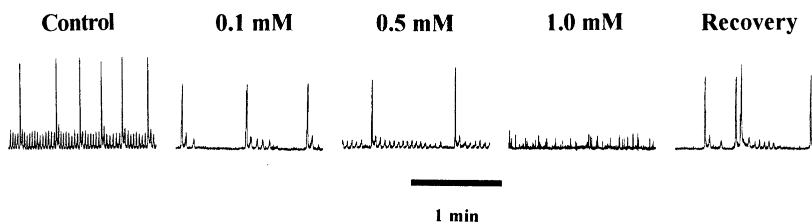

Fig. 3. Moving time-averaged respiratory-related neural activity recorded from the trigeminal nerve of the bullfrog brainstem perfused with control (1% DMSO in aCSF), 7-Nitroindazole (0.1, 0.5 and 1.0 mM) and approximately 60 min following 1.0 mM 7-NI perfusion in aCSF (recovery).

with the facilitatory effect of glutamate on respiratory-related neural activity in this preparation [24,30].

The results of our previous study show that the addition of SNP or L-Arg significantly increased respiratory-related burst frequency (Fig. 2). In most preparations, SNP caused a sustained increase of burst frequency over the 20-min perfusion, whereas L-Arg had more variable effects on neural activity including an inhibitory effect in some preparations. The general NOS inhibitor, L-NA, reversibly attenuates or abolishes fictive breathing (Fig. 2). SNP spontaneously generates NO extracellularly in the presence of oxygen whereas L-Arg produces NO intracellularly as the by-product of its conversion to L-citrulline by NOS [35]; therefore, tissue diffusivity may play an important role in the effectiveness of these compounds to affect neurons of the CPG. L-Arg generates intermediate compounds during its conversion to L-citrulline and, in addition to the presence of NOS, requires several cofactors to generate NO during this conversion. The variability in response to L-Arg perfusion in this study may be due to feedback inhibition limiting production of NO or inadequate amounts of cofactors in some preparations.

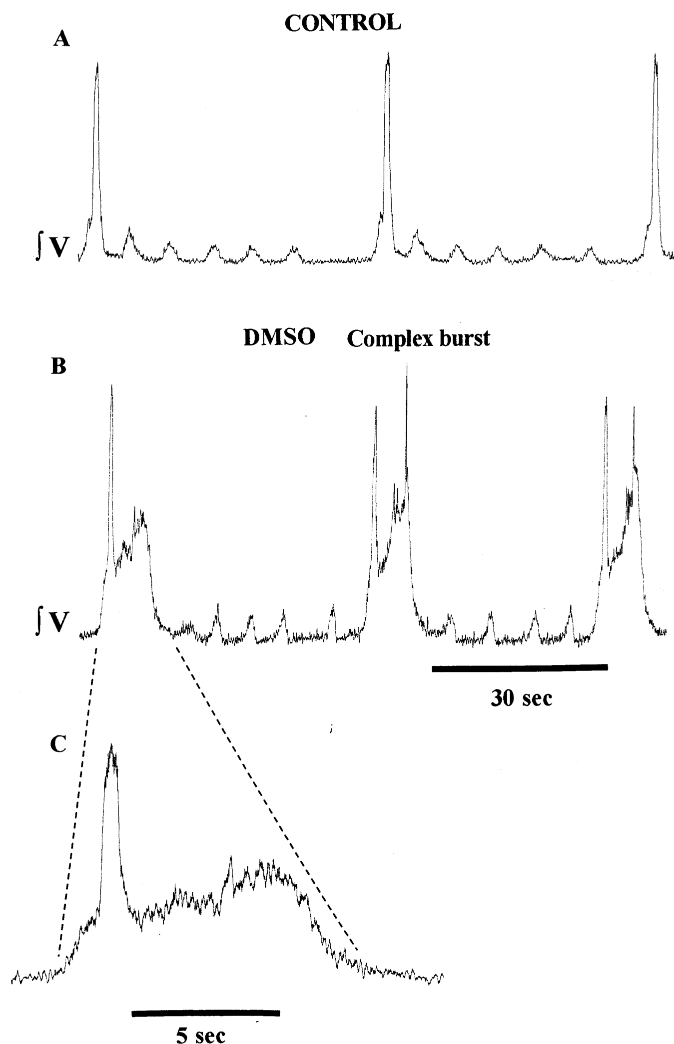

Fig. 4. (A) Moving time averaged activity recorded from the trigeminal nerve in control (aCSF). (B) Same preparation in 1% DMSO illustrating the increases in peak integrated activity and burst duration for a complex burst. (C) One complex burst from (B) recorded at a faster sweep speed.

Our study also implicates nNOS as the specific enzyme responsible for synthesis of NO and the respiratory effects of NO in the bullfrog brainstem. 7-Nitroindazole, a specific inhibitor of nNOS, reversibly inhibited respiratory-related burst frequency in a dose-dependent manner (Fig. 3; Table 1), but had no significant effect on respiratory variables associated with respiratory pattern formation, such as burst duration or burst amplitude (Fig. 6). In some experiments, DMSO, the vehicle for 7-NI, increased burst duration and amplitude (Figs. 4 and 5), but did not affect burst frequency (Table 1). DMSO has been shown to have stimulatory effects on respiration in mammals that are centrally mediated [8]. In our study, DMSO did not affect respiratory rhythm generation, but did have stimulatory effects on burst amplitude and duration in some experiments, suggesting that DMSO does not affect the mechanisms for respiratory rhythm generation but, instead, may affect neurons involved with pattern formation. It has been suggested that there are separate mechanisms for respiratory rhythm generation and burst pattern formation [11], but this is difficult to distinguish in the vertebrate brainstem where the respiratory CPG has not been clearly identified [31]. The effects of DMSO also appear to be attenuated by 7-NI since DMSO alone results in larger increases in burst duration and amplitude than when 7-NI is present (Fig. 5). Overall, our results suggest that NO has excitatory effects on neuronal properties related to CPG regulation of respiratory frequency, but not those neurons responsible for shaping respiratory bursts.

A recent study has demonstrated the presence of NADPH-diaphorase (ND), an intracellular marker for NOS, throughout the brainstem of *Rana perezi* [26]. That study demonstrated the presence of ND staining within the medulla which was primarily localized to the reticular formation, the proposed site of the neurons comprising the respiratory CPG in vertebrates [2,6,11]. The study by Munoz et al. [26] also showed that ND was not found in cranial motoneurons, suggesting that the effects of NO in the present study were not likely due to stimulation of motoneurons but, instead, stimulation of neurons linked to, or directly involved with, the respiratory CPG.

The results from the present study are also consistent with previous experiments that have examined a role for NO in breathing in mammals. Recent studies in awake rats have demonstrated that NO is important in the transduction of hypoxic stimuli in the CNS. For

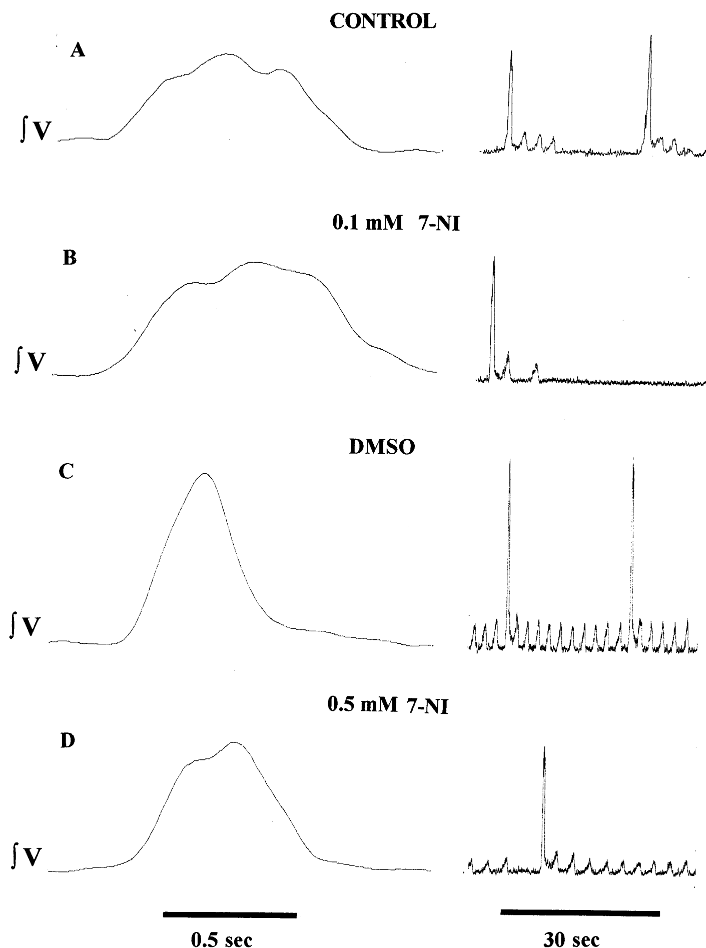

Fig. 5. (A) Moving time averaged activity recorded from the trigeminal nerve in control (aCSF) showing a single lung burst at fast sweep speed (left) and at slower sweep speed (right). (B) Perfusion with 0.1 mM 7-NI showing reduced lung burst frequency. (C) Perfusion with 1% DMSO (vehicle) slightly increases burst amplitude from control and stimulates buccal oscillations. (D) Perfusion with 0.5 mM 7-NI reduces lung burst frequency and amplitude from the DMSO perfusion. Increased concentration of 7-NI to 1.0 mM completely abolished buccal and lung neural activity (data not shown).

example, intravenous administration of a specific nNOS inhibitor, *S*-methyl-thiocitrulline, significantly attenuates hypoxic ventilatory reflexes in awake rats [15]. In addition, spontaneous neural activity recorded from brainstem slices containing a region containing the NTS, the first central relay for carotid chemoreceptor afferents, is significantly increased by bath application of L-Arg and significantly attenuated with the competitive NOS inhibitor L-NAME [38]. Taken together, results from the present study, and from mammalian studies, indicate that NO acts primarily as an excitatory neurotransmitter within the CNS with respect to ventilation.

In conscious adult amphibians, lung ventilation may be abolished with artificial, uni-directional ventilation (UDV) of the lungs with hypocapnic and/or hyperoxic gas mixtures [19,39]. This raises the question as to the origin of respiratory drive in the amphibian brainstem preparation that is superfused with aCSF equilibrated with hyperoxic (95–100% O_2) gas mixtures. The amphibian brainstem preparation is well oxygenated at the center of the brain tissue (e.g. Po_2 = ca 350 Torr) when superfused with hyperoxic gas mixtures [37]. Because NO production is dependent upon levels of brain tissue oxygenation [35], there may be significant amounts of NO in neural tissue at these high levels of Po_2 in this preparation. If NO acts as an excitatory neurotransmitter, as the data from the present study suggest, then the persistence of fictive breathing in the amphibian brainstem preparation during hyperoxic superfusion may be related to the levels of NO production in the tissue. In support of this suggestion, Gozal [14] recently demonstrated that a brief hyperoxic exposure potentiates the hypoxic ventilatory response in awake rats and that 7-NI abolished this potentiation. This result indicates that hyperoxia may activate nNOS and increase NO production which, in turn, potentiates the central component of the hypoxic ventilatory response. Whether the in vitro preparation is potentiated by elevated levels of NO production in neural tissue by hyperoxia remains to be examined.

Nitric oxide acts intracellularly on the heme moiety of guanylate cyclase and raises the intracellular concentration of cGMP in the cell in which it is produced. Owing to its lipophilicity and high diffusivity, NO can modulate cGMP production in neighboring cells; thus, NO can act as an *intra*cellular as well as an *inter*cellular neurotransmitter/neuromodulator. This has led to the suggestion that NO may subserve a synchronizing function within neural networks [1,27]. In support of this hypothesis, it has been shown that NO regulates oscillatory neural activity within thalamocortical neurons in the vertebrate brain [28]. The NO-cGMP pathway also activates CPG-mediated motor programs associated with olfaction [13], feeding [25] and feeding behavior [9] in terrestrial molluscs. Until recently, however, there has been little direct evidence for a nonsynaptic function for NO is mediating neural networks. Using cultured motoneurons from a terrestrial mollusc that are involved with feeding behavior and express NOS, Park et al. [29] have shown that NO mediates a slow excitatory anterograde transmission between adjacent neurons at distances up to 50 μm.

NO may be the earliest known biological signaling molecules [10]. It has been suggested that CPGs for a variety of motor behaviors, including breathing, may have been conserved in the course of evolution [36], despite the obvious diversity in structures associated with breathing. Implicit in this suggestion is that neurotransmitters associated with CPGs, such as NO, should also have co-evolved with networks that provide the neural substrate supporting motor behaviors. Our results indicating that NO is an important excitatory molecule involved in the regulation of the bullfrog respiratory CPG appear to support this hypothesis.

Because the particular location of the respiratory CPG and identifiable NOS-containing neurons in close proximity to these sites is unknown, further studies will have to be done to localize the effects of NO on central respiratory rhythm generating neurons in the bullfrog brainstem. Regardless of the precise location of the respiratory CPG, our results strongly suggest that endogenous production NO via nNOS is important for neurotransmission or neuromodulation of respiratory drive for breathing in the bullfrog brainstem.

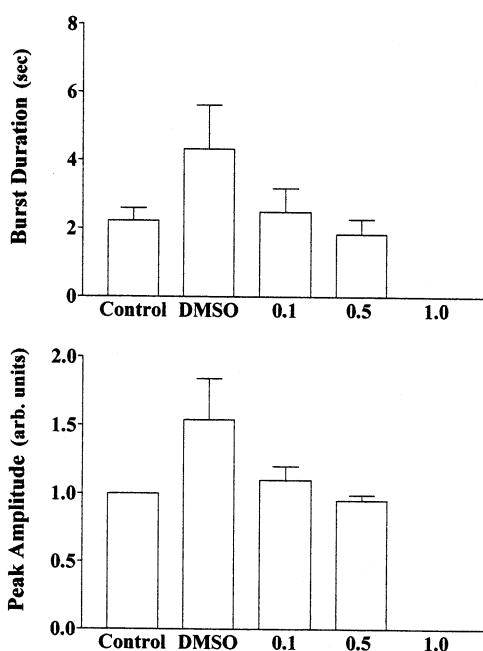

Fig. 6. Burst duration (s) and normalized peak integrated response for brainstem activity in control (aCSF), DMSO (1% in aCSF) and 7-NI (0.1, 0.5 and 1.0 mM) for seven *R. catesbeiana* in vitro preparations.

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