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Nitric oxide modulates respiratory-related neural activity in the isolated brainstem of the bullfrog

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Abstract

The effects of nitric oxide (NO) on respiratory-related neural activity were investigated using the isolated brainstem preparation from bullfrogs (*Rana catesbeiana*). Addition of the NO donor, sodium nitroprusside (SNP), or the amino acid precursor for NO synthesis, L-arginine (L-Arg), produced significant increases in respiratory-related burst frequency. Inhibition of nitric oxide synthase (NOS) with N^{ω}-nitro-L-arginine (L-NA), a non-selective NOS inhibitor, 7-nitro indazole (7-NI), reversibly abolished burst activity. These results suggest that production of NO, probably via neuronal NOS (nNOS), provides a facilitatory input to the respiratory central pattern generator (CPG) in the amphibian brainstem. Endogenous production of NO may be a necessary inter- or intracellular messenger for neurotransmission and/or neuromodulation of central respiratory drive to motor effecters in the bullfrog. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved

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Breathing in vertebrates is an autonomic process that originates with an oscillatory central pattern generator (CPG) in the reticular formation of the brainstem [3]. Although the respiratory CPG has not been precisely identified, its characteristics have been studied extensively with in vitro brainstem preparations from a number of vertebrates [6,10,17,18]. Since phylogenetically diverse animals show similarities with respect to central respiratory rhythmogenesis in these reduced preparations, it has been hypothesised that central neural networks for breathing and other functions may be evolutionarily conserved [19]. In this context, it would seem likely that evolutionarily-conserved neurotransmitters, such as nitric oxide (NO) [5], might be important with regard to regulation of central pattern generators.

Nitric oxide is a free radical gas that has been shown to be involved in many diverse physiological processes in the central and peripheral nervous systems of vertebrates and invertebrates [7]. Owing to its unique properties as a lipophilic messenger molecule, NO may act as an intercellular, as well as intracellular, neurotransmitter; thus, it has been suggested that NO may be capable of performing a 'synchronizing' role for neural network oscillators in the nervous systems of invertebrates and vertebrates [1,15].

The role of NO in the control of breathing is unclear. In cats, NO appears to be necessary for a pontine phase-switching function [11], however, more recent experiments in mammals suggest that NO is an excitatory neurotransmitter in the CNS [8] and is important for the transduction of hypoxic stimuli [9]. At present, modulation of the respiratory CPG by NO in vertebrates has not been examined. 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*).

A total of 26 bullfrogs (100–190 g) were used for 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

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ceased (ca. 15–20 min). The animal was then quickly 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 ice-cold (5–7°C), oxygenated artificial CSF (aCSF) with a composition used in a previous study with this species [10]. 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 rootless 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 [17]. The vagus nerve innervates muscles of the glottis which controls the 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 [10,12]. Neural activity was amplified 10 000 times with 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, or directly onto computer 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 seven additional preparations, 7-NI (0.1, 0.5 and 1.0 mM) was used to examine the effects of blockade of nNOS on respiratory-related neural activity. 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 solitaries (NTS) in rats [20]. All drugs were obtained from Sigma Chemicals (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 rhythm had returned close to its original pre-drug rate. Thus, the respiratory rate during perfusion with each drug solution was compared with its own rate immediately prior to switching to the test solution. Significant differences in respiratory burst frequency between control and test solutions were determined by paired *t*-test. Significance was taken as $P < 0.05$.

Table 1

Effects of nitrergic drugs on respiratory-related neural activity (bursts/min) in the bullfrog brainstem preparation in vitro

Drug (mM)	Control	Test
SNP	2.0 ± 0.3	3.4 ± 0.4 (14)**
l-Arg	1.5 ± 0.3	2.3 ± 0.3 (13)*
l-NA	1.7 ± 0.2	0.3 ± 0.2 (15)***

Values are mean ± SE (no. of preparations). Burst frequency measured between 10–20 min of brainstem perfusion with aCSF containing one test solution (drug) and compared with control (pre-infusion aCSF) values by paired *t*-test. *$P < 0.05$ **$P < 0.002$: ***$P < 0.001$.

Burst frequency varied between 0.7 and 5 bursts/min with an overall average of 1.7 ± 0.3 ($n = 26$) during perfusion with oxygenated, isocapnic aCSF. These rates are within the range of frequencies found in a previous study using this species [12]. A summary of the effects of SNP, L-Arg and L-NA administration on burst frequency is given in Table 1. 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 ($t_{13} = 4.2$; $P < 0.002$; Table 1). The effect was entirely reversible in all preparations. L-Arg perfusion resulted in a 65% increase in burst frequency ($t_{12} = 2.41$; $P < 0.05$; Table 1), but there was more variability associated with L-Arg perfusion including an attenuation of burst frequency in four of 13 preparations. Higher concentrations (10 mM) of SNP or L-Arg resulted in large increases in burst frequency. Fig. 1A illustrates the effects of a moderate (1 mM) and a high (10 mM) concentration of SNP and L-Arg on neural activity in one preparation.

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 ($t_{14} = 5.58$; $P < 0.001$; Table 1); at these concentrations neural activity

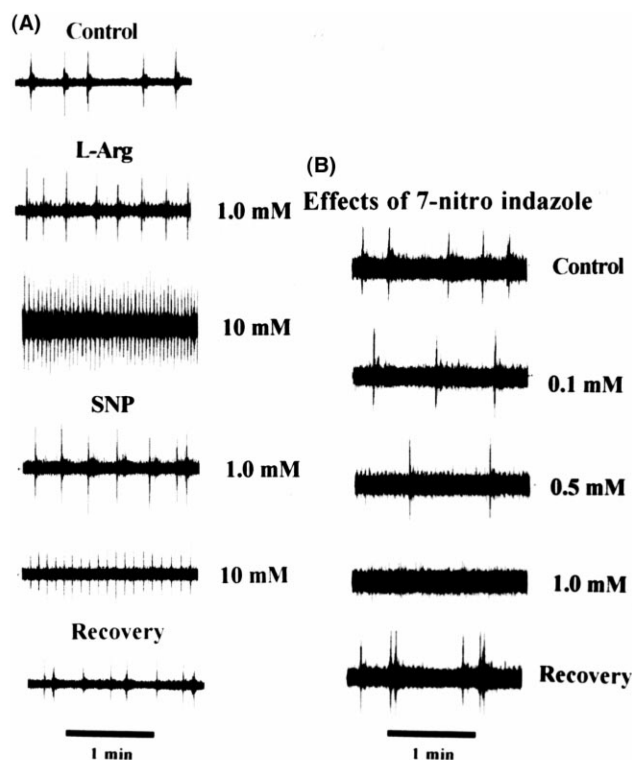

Fig. 1. (A). Respiratory-related neural activity recorded from the hypoglossal nerve of the bullfrog brainstem in vitro during aCSF (control), l-Arg (0.1 and 10 mM) and SNP (0.1 and 10 mM) administration and following SNP perfusion (recovery). (B). Respiratory-related neural activity recorded from the trigeminal nerve of the bullfrog brainstem perfused during aCSF (control), 7-Ni (0.1, 0.5 and 1.0 mM) administration and approximately 60 min following 1.0 mM 7-NI (recovery).

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 and reversibly abolished neural activity in all 7 preparations at 1 mM (Figs. 1B and 2). At the highest concentration of 7-NI used (1 mM), burst activity resumed approximately 1 h after returning to aCSF perfusion (Fig. 1B).

This study has shown that endogenous NO production, principally via nNOS, modulates the respiratory CPG in the isolated brainstem of the bullfrog. The selective nNOS inhibitor 7-NI produced a significant, dose-dependent reduction of respiratory-related frequency suggesting that endogenously produced NO acts as an excitatory neurotransmitter/neuromodulator within the CNS of the bullfrog. Although L-NA also abolished neural activity, higher doses were required to achieve this effect compared with 7-NI. We attribute this to either the ease with which 7-NI may cross cell membranes and diffuses to the site(s) of action and/or its higher specificity for nNOS compared with L-NA.

A recent study has demonstrated the presence of NADPH-diaphorase (ND), an intracellular marker for NOS, throughout the brainstem of *Rana perezi* [14]. Their study showed that ND staining within the medulla was primarily localized to the reticular formation, the proposed site of the neurons comprising the respiratory CPG in vertebrates [3]. Their study 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.

Previous experiments with the bullfrog brainstem preparation have shown that microinjection of glutamate into rostral areas of the medullary reticular formation significantly increases respiratory burst frequency, whereas microinjections at more caudal locations inhibits neural output or has no effect [12]. Their study did not identify specific receptors that mediated 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 [2]. In the frog brainstem preparation, the link between NMDA receptor stimulation and NO production has not been conclusively established, but our results are consistent with the facilitatory effect of glutamate on respiratory-related neural activity in this preparation [12].

In the present study, addition of SNP or L-Arg significantly increased respiratory-related burst frequency. 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. SNP spontaneously generates NO extracellularly in the presence of oxygen whereas L-Arg produces NO intracellularly as the byproduct of its conversion to L-citrulline by NOS [7]; 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

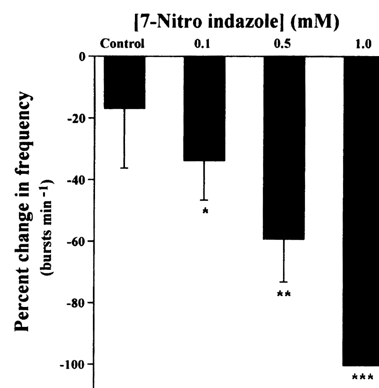

Fig. 2. Dose-dependent reductions in mean (±SE) burst frequency (bursts/min) during control (1% DMSO in aCSF) perfusion or during 7-NI administration at 0.1, 0.5 and 1.0 mM. Values represent the percent change between pre-perfusion burst frequency and the frequency obtained during the 10–20 min period of 7-NI perfusion or 1% DMSO control ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

amounts of cofactors in some preparations. Increased perfusate concentration of L-Arg or SNP to 10 mM in this preparation resulted in an increase of burst frequency (Fig. 1A), which is consistent with the hypothesis that feedback inhibition or inadequate amounts of cofactors resulted in inhibitor of neural activity at lower concentrations.

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 example. intravenous administration of a specific nNOS inhibitor, *S*-methyl-thiocitrulline, significantly attenuates hypoxic ventilatory reflexes in awake rats [8]. 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 [20]. 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.

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 intracellular as well as an intercellular neurotransmitter/neuromodulator. This has led to the suggestion that NO may subserve a synchronizing function within neural networks [1,15]. In support of this hypothesis, it has been shown that NO regulates oscillatory neural activity within thalamocortical neurons [16]. The NO-cGMP pathway also activates CPG-mediated motor programs associated with feeding [13] and feeding behavior in molluscs [4].

It has been suggested that CPGs for a variety of motor behaviors, including breathing, may have been conserved in the course of evolution [19], 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 appears to support this hypothesis.

Since 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 neurotransmisson or neuromodulation of respiratory drive for breathing in the bullfrog brainstem.

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