Central respiratory activity of the tadpole in vitro brain stem is modulated diversely by nitric oxide

MICHAEL B. HARRIS, RICHARD J. A. WILSON, KONSTANTINON VASILAKOS, BARBARA E. TAYLOR, AND JOHN E. REMMERS. Central respiratory activity of the tadpole in vitro brain stem is modulated diversely by nitric oxide. Am J Physiol Regulatory Integrative Comp Physiol 283: R417–R428, 2002. First published May 6, 2002; 10.1152/ajpregu.00513.2001.—Nitric oxide (NO) is a potent central neuromodulator of respiration, yet its scope and site of action are unclear. We used 7-nitroindazole (7-NI), a selective inhibitor of endogenous neuronal NO synthesis, to investigate the neurogenesis of respiration in larval bullfrog (Rana catesbeiana) isolated brain stems. 7-NI treatment (0.0625–0.75 mM) increased the specific frequency of buccal ventilation (BV) events, indicating influence on BV central rhythm generators (CRGs). The drug reduced occurrence, altered burst shape, and disrupted clustering of lung ventilation (LV) events, without altering their specific frequency. LV burst occurrence and clustering also differed between pH conditions. We conclude that NO has diverse effects on respiratory rhythmogenesis, being necessary for the expression of respiratory rhythms, inhibiting the frequency of BV CRG, and affecting both shape and clustering of LV bursts through conditional modulation of LV CRG. We confirm central chemosensitivity in these preparations and demonstrate chemomodulation of LV burst clustering and occurrence but not specific frequency. Results support distinct oscillators underlying LV and BV CRGs.

NITRIC OXIDE (NO) is a potent chemical messenger in the central nervous system. It is synthesized in neurons via a constitutive and calcium-sensitive isoform of the enzyme NO synthase [NOS-1 or neuronal NOS (nNOS); for review, see Refs. 11, 18]. Neuronal NO synthesis is induced, in part, by the synaptic activation of N-methyl-D-aspartate-type glutamatergic receptors as well as by GABA and β-endorphins. The glutamatergic neuromodulatory cascade is intimately involved in the genesis and regulation of breathing in vertebrates, and a growing body of evidence suggests that NO is an extremely important neuromodulator of central respiratory control (3, 10, 12, 15–17, 19, 20, 29, 64). The specific respiratory action of NO, however, remains largely unexplored.

In vitro brain stem preparations are used extensively to examine the central mechanisms responsible for the generation and modulation of respiratory rhythm in vertebrates (37, 44, 48, 49, 58). Such preparations derived from ectothermic vertebrates benefit from their small dimension, relative hypoxia tolerance, and low metabolic demand (22, 28, 34, 44, 53, 54, 68). In vitro brain stem preparations derived from larval amphibians, being well oxygenated and relatively non-acidic, are particularly well suited to investigations of vertebrate central respiratory rhythmogenesis and pattern formation (32, 44, 61, 69).

Semiterrestrial (postmetamorphic) tadpoles and adult frogs such as Rana catesbeiana exhibit prolonged periods of rhythmic ventilation of the buccal cavity, termed buccal oscillations, interspersed by tidal lung ventilation (LV), which occurs as either single events or periodic multibreath clusters (6, 7, 9, 13, 24, 26, 51, 67). Motor patterns indicative of both buccal ventilation (BV) and LV persist in the amphibian in vitro brain stem preparation, indicating that the capacity for their generation resides within the brain itself (28, 34, 53, 54). The neuronal elements that generate these ventilatory patterns and the factors that modulate the period and expression of these behaviors are poorly understood.

Hedrick and co-workers (19, 20), using an in vitro preparation derived from adult bullfrogs, have recently demonstrated that NO stimulates the occurrence of LV and propose that NO may be a necessary messenger for neurotransmission and/or modulation of central respiratory drive. Indeed, these authors suggest that the general incidence of such robust fictive breathing among in vitro preparations may result from an exci-
tatory influence of NO (19). The influence of NO on ventilatory pattern formation, and the levels at which it acts, however, are unclear. In the present study, we used in vitro brain stem preparations derived from larval bullfrogs to further investigate the role of NO, synthesized via nNOS, in the genesis and modulation of respiratory rhythm. Specifically, we address the role of NO beyond the modulation of lung ventilatory occurrence. We examine the specific frequency (the inverse of the interval between successive events) of respiratory burst discharge, taken to represent the activity of respiratory central rhythm generators (CRGs), as well as the overall occurrence rate (events per unit time) of this discharge and the shape and tendency of these LV bursts to occur in periodic clusters, reflecting the conditional modulation of CRG activity. We postulate that endogenously synthesized NO, arising from nNOS, influences the inherent frequency as well as the conditional expression of central respiratory activity.

METHODS

A total of 19 postmetamorphic R. catesbeiana tadpoles (stages 23–25, Taylor-Koliros scheme, Ref. 60) were used in this investigation. Animals were purchased from a commercial supplier and maintained at room temperature in filtered dechlorinated water. This research was approved by the institutional animal care and use committee, and animal care and experimental protocols conformed to local and national standards of ethics. The work fully conforms with the Guiding Principles for Research Involving Animals and Human Beings specified by the American Physiological Society.

Surgical Preparation

Animals were initially anesthetized by immersion in an ice-cold 0.2 mM solution of tricaine methanesulfonate (MS222; Sigma) in dechlorinated water, buffered with NaHCO₃ to pH 7.4, until unresponsive to tail or leg pinch (30–60 s). A block of tissue spanning a region between the orbits and forelimbs dorsal to the mouth was removed and placed in a dissection chamber, where the dorsal arachnoid membrane on the ventral medulla were removed. This decerebration was achieved within 2 min of initiating dissection. During subsequent dissection, brain stems were superfused with ice-cold oxygenated artificial cerebrospinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 MgCl₂, 10 d-glucose, 25 NaHCO₃, and 2.4 CaCl₂, equilibrated with 100% O₂.

Surgical procedures for preparing the in vitro brain stem have been described previously (61, 62). Briefly, the brain was transected midtectum, at the level of the third cranial nerve (CN III) and 2 to 4 mm caudal to the second spinal nerve (SN II). The choroid plexus, dura, and portions of the arachnoid membrane on the ventral medulla were removed. Brain stems were then transferred to a low-volume (0.5 ml) flow-through recording chamber (61, 62). Preparations were supported ventral side up between coarse nylon mesh such that all sides were bathed with aCSF flowing from rostral to caudal over the tissue at a rate of 5–10 ml/min, resulting in 15–30 dish volume changes per minute. The aCSF solution, noted above, closely matches the ionic composition reported for postmetamorphic tadpole and adult frog plasma (6, 24, 57). In particular, the aCSF bicarbonate concentration is equal to that of plasma, so that equilibration of aCSF with a normal CO₂ produced a normal pH. Preparations maintained in this solution produce easily identifiable respiratory discharge patterns that are consistent and stable for many hours and that closely resemble those associated with spontaneous ventilatory behavior in vivo (13). A supply of aCSF flowed to the chamber from perfusion reservoirs through glass tubing and a bubble trap. The reservoirs served as tonometers where aCSF was equilibrated with O₂-CO₂ gas mixtures to produce the desired pH. The fractional concentration of O₂ and CO₂ (F₀₂ and F.CO₂, respectively) in the equilibration gas was regulated with precision needle-valve flow meters on the in line, and F.CO₂ was monitored with an infrared medical gas analyzer (SensorMedics, LB-2). The pH of the solution in the tonometer was monitored (Corning 140) and maintained between 7.6 and 8.0 by adjusting F.CO₂ in the equilibration gas. Solutions were equilibrated with approximately 3.5, 2.2, and 1.4% CO₂, balance O₂, to produce aCSF pH of 7.6, 7.8, and 8.0, respectively. The resulting aCSF CO₂ partial pressures were approximately 25, 16, and 10 mmHg, respectively. Pilot studies demonstrated that the pH of the tonometers and recording chamber were identical and stabilized in 2 to 3 min after a change in equilibration gas F.CO₂.

Nerve Recording

Roots of CN VII and SN II were drawn into glass suction electrodes fabricated from 1-mm-diameter capillary glass (A-M Systems, Everett, WA) pulled to tip diameters of ~40 μm. Whole nerve discharge was amplified (~×1,000) and filtered (100–300 Hz [low pass] to 1 kHz [high pass]) using a differential AC amplifier (model 1700, A-M Systems) and fed to a modified Bessel filter (time constant 50 ms; SagaTech, Calgary, Alberta, Canada; see Ref. 45). Both raw (amplified) and Bessel-filtered signals were digitized at 2,000 Hz/channel and archived as computer files (AT-CODAS, DATAQ Instruments, Akron, OH) for subsequent analysis (ADVPPOST and WINDAQ, DATAQ Instruments).

Burst Classification

The burst discharge patterns of various cranial and spinal nerves associated with the normal activation of respiratory muscles during spontaneous LV, and either gill ventilation or buccal oscillations, have been previously characterized in both frogs and tadpoles (13, 25, 30, 51, 52). Burst activity patterns recorded from CN VII and SN II are indicative of the activation of muscles that contract in phase with movements of the buccal cavity during spontaneous ventilation, such as the depressor mandibulae, subhyoideus, sternohyoid, hyoglossus, and omohyoid muscles (51). Burst activity patterns were classified as described by Torgerson et al. (62) and Gdovin et al. (13) as corresponding to buccal ventilation (BV) and LV. BV bursts were identified as rhythmic bursts of activity in CN VII recordings, of <1 s in duration and having a generally monophasic gradual incrementing onset and decrementing offset profile. The relative amplitude of BV bursts in the Bessel-filtered signal was generally 3 to 5 times that of tonic baseline activity. BV bursts were apparent, frequently but not always, in the SN II neurogram. LV bursts were identified as rhythmic bursts of activity that were always coincident in CN VII and SN II recordings. Such bursts were also of <1 s in duration, were either monophasic or biphasic, and also had incrementing onset and decrementing offset profiles. The relative amplitude of LV bursts in the Bessel-filtered signal was generally 3 to 5 times that of BV bursts. Patterns of activity that did not meet the criterion for BV or LV bursts were classified as “nonrespiratory” (see Ref. 47 for examples). These bursts have long durations, large ampli-
tudes, or shapes inconsistent with those normally associated with ventilation, and they may represent neural activity associated with such behaviors as vocalization, feeding, swallowing, coughing, regurgitation, or gasping. Alternately, this discharge may not relate to naturally occurring phenomena in vivo and may be an artificial construct of some in vitro preparations. Such nonrespiratory bursts occurred uncommonly in our preparations.

Protocol

Disruption of endogenous NO synthesis. After the 1-h period of stabilization, Fco2 of the gas equilibrating the tonometer of aCSF was adjusted to produce an aCSF pH of either 7.6 (n = 5), 7.8 (n = 5), or 8.0 (n = 7), and burst activity was monitored for a 60-min baseline period. Pilot studies indicated that burst activity stabilized within ~10 min of a pH change. Preparations were then exposed to drug solutions containing various concentrations (0.06–1.0 mM) of a selective noncompetitive inhibitor of nNOS, 7-nitroindazole (7-NI), in aCSF. Drug solutions were prepared in identical tonometers, equilibrated with the same gas, and had the same pH as the baseline aCSF. Each preparation was exposed to at least four concentrations of 7-NI. The maximum 7-NI concentration was that which all but abolished LV burst activity during pilot investigations. As preparations produced different levels of activity at the three pH levels, the tested range of 7-NI concentrations differed between pH conditions. All preparations tested at a given pH were exposed to the same range of 7-NI concentrations. Treatment 7-NI concentrations were administered randomly, and each 30-min period of exposure was followed by at least a 30-min “washout” period of exposure to drug-free aCSF. Although levels and patterns of activity during washout periods returned toward those observed during initial conditions, most of the values obtained from such periods were different from initial values, indicating that residual effects of 7-NI exposure were long-lasting. Hedrick and Morales (19) also observed a long latency to incomplete recovery after exposure to 7-NI.

Data analysis. Data were analyzed from 15-min “observation” periods taken from the end of both the 60-min baseline and 30-min drug exposure periods. Values obtained from “washout” periods have not been included in the present analysis.

Burst occurrence rate and specific frequency. Total burst occurrence was the sum of all BV and LV bursts within the observation period. Burst occurrence rate was calculated as the ratio of total burst occurrence to observation period duration to yield an overall frequency (bursts/min). The specific frequency of bursts was calculated from the period of successive BV or LV bursts. Only pairs of sequential and alike bursts not separated by any other burst type were included in this analysis. The inverse of this period was multiplied by 60 and expressed as a frequency (bursts/min).

Burst duration and shape. The duration of BV and LV bursts was determined from the Bessel-filtered record of CN VII whole nerve discharge, as the time between burst onset, i.e., where the signal deviated from tonic background activity, to the point where the signal returned to this baseline. The shape of LV bursts in the Bessel-filtered CN VII discharge was assessed subjectively as being either biphasic or monophasic. The influences of NOS inhibition were demonstrated by generating event-triggered average tracings from individual preparations. Recordings of Bessel-filtered CN VII discharge surrounding single breaths or clusters were compiled from initial and treatment conditions. Tracings from 25 consecutive events from initial and treatment conditions were averaged and expressed as the mean level of activity ± 1 SD of the mean for each condition.

Burst clustering. In all cases, LV bursts occurred intermittently, with LV bursts occurring as periodic events (episodes) with otherwise relatively rhythmic and continuous BV burst activity. An episode of LV bursts was defined as either a single or an uninterrupted cluster of LV bursts separated from subsequent LV bursts by one or more BV bursts or, in cases where BV activity was absent, by a period exceeding twice the control LV-LV burst period. The clustering of LV bursts was assessed by determining the average number of bursts occurring in each episode, expressed as bursts/cluster, with single isolated bursts counted as a “cluster” of one burst for the purpose of analysis. The duration of burst episodes (the period from the onset of the first to the end of the last burst in an episode) and the number of episodes occurring during the observation period, expressed as episodes per minute, were also determined.

Statistical assessment. Statistical analyses were performed with the aid of a computer and statistical software (SigmaStat v. 2.03, Jandel Scientific). Influences of pH on burst discharge parameters under initial conditions were determined by a series of one-way (non-repeated) analyses of variance (ANOVA). The influence of 7-NI treatment was assessed through a series of one-way repeated-measures ANOVA for each pH condition. The specific frequency of BV and LV bursts was compared between pH conditions by a series of two-way (non-repeated) ANOVA made at each concentration of 7-NI. In these cases, additional pairwise multiple comparison procedures were done using the Student-Newman-Keuls method. Data illustrating the influence of pH on burst clustering under initial conditions were not normally distributed, and the parametric ANOVA assessing this influence did not achieve the desired power. As such, an additional analysis was made using a Kruskal-Wallis one-way ANOVA on ranks with Dunn’s method of pairwise multiple comparisons. In addition, a parametric t-test was used to assess the normally distributed subset of these data (between pH 7.8 and 8.0), as noted in the text. Data illustrating the influence of 7-NI on burst clustering at pH 7.6 were also not normally distributed, and parametric ANOVA assessing this influence did not achieve the desired power. Additional analysis was made using a Friedman repeated-measures ANOVA on ranks. All tests employed an α-value of 0.05; thus normality, equality of variance, and significance of differences were attributed to P < 0.05.

RESULTS

LV and BV Burst Discharge

For the most part, patterns of whole nerve burst discharge recorded from CN VII and SN II matched the criterion for classification as either LV or BV bursts. Under initial conditions, burst discharge pattern and shape were remarkably consistent both over time within a given preparation, and between preparations. At most, one or two nonrespiratory bursts occurred in any given 15-min period. As such, events were overshadowed by the far more prevalent LV and BV and were not analyzed. BV bursts were most prevalent, appearing to occur rhythmically and relatively continuously, interrupted by intermittent periods of LV burst activity (Fig. 1, top tracing).
Influence of NOS Disruption

Specific frequency. The specific frequency of LV bursts within a cluster was constant regardless of pH or the concentration of 7-NI (Fig. 2). The specific frequency of BV bursts, however, increased with 7-NI treatment ($F_{4.19} = 4.2, P = 0.03; F_{4.17} = 3.8, P = 0.04; F_{4.25} = 6.3, P = 0.04$, at pH 7.6, 7.8, and 8.0 respectively). Under no circumstance did the specific frequency of BV bursts exceed that of LV bursts.

Burst occurrence. Under all three pH conditions (7.6, 7.8, and 8.0), NOS inhibition influenced both LV and BV bursts. This treatment reduced the occurrence of LV bursts ($F_{4.24} = 5.6, P = 0.05; F_{5.20} = 22.6, P < 0.001; F_{4.29} = 11.6, P < 0.001$, respectively) and increased the occurrence of BV bursts ($F_{4.24} = 23.4, P < 0.001; F_{5.19} = 5.1, P = 0.014; F_{4.25} = 11.3, P < 0.001$, respectively, Fig. 3).

NOS inhibition tended to reduce the occurrence of LV bursts at all levels of perfusate acidity. However, LV bursts were more resilient to the effects of 7-NI at low pH. Thus, at the lowest level of pH (7.6), LV bursts were eliminated only at 7-NI concentrations of 1.0 mM. For pH values of 7.8 and 8.0, LV bursts were abolished with 7-NI concentrations above 0.75 and 0.5 mM, respectively. BV bursts were generally not discernable at 1.0 mM 7-NI. BV burst activity was robust below this level, and such bursts routinely persisted despite the relative absence of LV burst.

Burst shape. 7-NI treatment decreased LV burst duration only at pH 7.6 ($F_{4.24} = 6.3, P = 0.003$), while this treatment decreased BV burst duration at pH 7.6 and 8.0 ($F_{4.20} = 6.3, P = 0.006; F_{4.27} = 3.4, P = 0.033$, respectively, Fig. 4).

The shapes of LV bursts recorded from CN VII were generally biphasic, although a minority of isolated single bursts and occasionally the first bursts in a multiburst cluster were monophasic. NOS inhibition had an obvious influence on this shape by causing a dose-dependent reduction in the magnitude of the initial phase of the biphasic burst and a smoothing of the initial peak (Figs. 5 and 6). This resulted in a more gradual and smaller initial wave, which resembled the rising phase of a BV burst, followed by a monophasic peak.

Clustering LV bursts into multiburst episodes. 7-NI treatment altered the number of LV bursts occurring in each episode (Fig. 7). At pH 7.6, 7-NI treatment caused a marginal reduction in the number of bursts per cluster (Fig. 7B, $F_{4.24} = 2.9, P = 0.051$). However, the power of this assessment ($0.457$ at $\alpha = 0.05$) was less than the 0.800 desired, and data were not normally distributed. The trend for a decrease in the number of bursts per cluster with 7-NI was demonstrated by a Friedman repeated-measures ANOVA on ranks (Chi-square = 17.053 with 4 degrees of freedom; $P = 0.002$). At pH 7.8, 7-NI treatment reduced the number of bursts per cluster and converted multiburst episodes to single bursts ($F_{5.20} = 15.0, P < 0.001$; Fig. 7C). LV bursts at pH 8.0 generally occurred as single or paired bursts per episode. NOS inhibition altered burst clustering at pH 8.0 ($F_{4.22} = 4.9, P = 0.014$), at low doses by enhancing the number of bursts per cluster ($P = 0.018$ and 0.036 by multiple comparison at 0.06 and 0.12 mM, respectively) and at higher doses by reducing them (Fig. 7D).

Importantly, 7-NI treatment could independently alter clustering without changing burst occurrence (Fig. 7). At pH 7.8, 0.12 mM 7-NI reduced the number of bursts per cluster without changing overall LV burst occurrence. Figure 1 illustrates a particular case where 7-NI treatment clearly converts burst pattern from multiburst episodes to single unclustered bursts, with-

![Fig. 1. Recordings of the moving average of whole nerve discharge in cranial nerve VII (CN VII) illustrating ventilatory motor patterns from an isolated tadpole brain stem bathed in pH 7.6 mock cerebral spinal fluid with various concentrations of 7-nitroindazole (7-NI), in mM. Two distinct motor patterns are apparent: large and small amplitude bursts, indicative of lung and buccal ventilation, respectively.](image-url)
out altering overall burst occurrence (between 0 and 0.5 mM 7-NI). The apparent reductions in overall LV burst occurrence caused by 0.25 mM 7-NI at pH 8.0 with no change in clustering (Fig. 7D) reflects simply the fact that in these cases bursts generally occurred as single events rather than multiburst clusters.

NOS inhibition did not alter the frequency of LV burst episodes. The duration of such episodes at pH 7.6 and 7.8 was reduced by 7-NI treatment ($F_{4,24} = 3.407$, $P = 0.034$; $F_{5,21} = 6.55$, $P = 0.004$), although no similar effects were noted at pH 8.0 ($F_{4,22} = 1.375$, $P = 0.30$; power at $\alpha = 0.050$: 0.107 < 0.800).

**Influence of CO$_2$/H$^+$ in Drug-Free Superfusate**

Specific frequency. LV burst specific frequency (within a cluster of breaths) was insensitive to changes in pH, while the specific frequency of BV bursts was influenced by pH ($F_{2,16} = 7.5$, $P = 0.006$, Fig. 2), being higher at pH 7.8 than either 7.6 or 8.0 ($P = 0.05$ and 0.038). Regardless of pH, however, BV burst specific frequency was always lower than that of LV bursts ($F_{2,33} = 19.0$, $P < 0.001$). The duration of LV bursts increased with elevations in pH ($F_{2,16} = 4.8$, $P = 0.027$), with longer bursts occurring at pH 8.0 than pH
7.6 ($P = 0.026$), while pH did not influence the duration of BV bursts (Fig. 4).

**Burst occurrence.** There was a significant influence of pH on the occurrence of lung and buccal bursts ($F_{2,16} = 7.1, P = 0.008; F_{2,16} = 5.0, P = 0.024$, respectively, Fig. 3). Fewer LV bursts occurred at pH 8.0 than at either pH 7.8 or 7.6 ($P = 0.024$ and 0.008, respectively), while the values at pH 7.8 and 7.6 did not differ significantly. Fewer BV bursts occurred at pH 7.6 than at either pH 7.8 or 8.0 ($P = 0.031$ and 0.02) with no differences between pH 7.8 and 8.0 ($P = 0.637$; power at $\alpha = 0.050$: 0.609 < 0.800).

**Clustering bursts.** LV bursts occurred intermittently as episodes of either single or clustered LV bursts, each separated by BV bursts. CO$_2$/H$^+$ did not consistently influence the tendency to cluster bursts over the full range of pH levels ($F_{2,16} = 2.16, P = 0.152$, by one-way ANOVA), although the statistical power of this assessment (0.208 at $\alpha = 0.050$) was well below that desired (0.800), and data were not normally distributed. The trend for an increase in the number of bursts per cluster with a decrease in pH was demonstrated by a nonparametric Kruskal-Wallis one-way ANOVA on ranks ($H = 6.633$ with 2 degrees of freedom, $P = 0.036$). The greater predominance of single LV bursts and fewer LV bursts occurring per cluster at pH 8.0 than at pH 7.8 was demonstrated by Dunn’s pairwise multiple comparison procedure (difference on ranks =

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**Fig. 3.** Histograms illustrating the effect of 7-NI on the occurrence (means $\pm$ SE, expressed as bursts/min) of lung and buccal respiratory events, during exposure to various concentrations of 7-NI in superfusates with pH 7.6 (A), 7.8 (B), and 8.0 (C). *Significant difference from values observed during initial exposure to drug-free superfusate ($P < 0.05$).
7.243; $Q = 2.45; P < 0.05$) or by parametric $t$-test ($t = 5.159$ with 10 degrees of freedom, $P < 0.001$; power at $\alpha = 0.050: 0.997$; Fig. 7A). Changes in burst occurrence were generally associated with corresponding changes in clustering. No influence of pH on the number of LV burst episodes per minute was detected, although the duration of these episodes was marginally influenced by pH ($F_{2.16} = 3.5, P = 0.058$; power at $\alpha = 0.050: 0.584 < 0.800$; Fig. 4).

**DISCUSSION**

**General**

LV bursts in the amphibian brain stem preparation occur in clusters separated by periods of BV. We have attempted to elucidate the effects of 7-NI and of changes in $\text{PCO}_2$/pH by examining overall rate of burst occurrence, the number of LV bursts per episode, and the specific frequency of LV and BV bursts. The specific frequencies of sequential ventilatory events represent the period or oscillatory frequency of the CRG(s) controlling these events. This variable indicates the endogenous rhythm of intermittent events (28). While changes in the apparent frequency of bursts can occur through skipped beats, changes in specific frequency, in the absence of skipped beats, can result only from direct modulation of mechanisms responsible for rhythmogenesis. In contrast, overall burst occurrence is more indicative of the degree to which the
conditional oscillator is active. Changes in the occurrence of cranial nerve bursts can result from changes in the frequency of a central oscillator, but can also result from upstream modulation of the conditional oscillator, which alters the probability of its activation or quiescence, as well as downstream modulation of the transduction of oscillator output at the level of premotor and motor neuron pools.

Sensitivity of In Vitro Ventilatory Pattern to NOS Disruption

Specific frequency of ventilatory bursts. The specific frequency of LV bursts within clusters was invariant over the range of 7-NI treatment, indicating that endogenous NO does not regulate the frequency of the oscillator producing this burst pattern. In contrast, the elevation of BV specific frequency with 7-NI treatment suggests that the oscillator responsible for BV burst generation is frequency modulated by NO. These results suggest that the period of the BV oscillator is normally depressed by endogenous NO synthesis and that different circuits generate these two types of rhythm.

The genesis of buccal oscillations appears to require postsynaptic inhibition, while lung oscillations do not, suggesting that the buccal oscillator requires network interaction while the lung oscillator possesses pacemaker properties (8). NO does not appear to influence the pacemaker properties of the lung oscillator. As disruption of endogenous NO synthesis increased the specific frequency of BV bursts, our present results suggest that endogenous NO lengthens the period of the buccal oscillator, possibly by modulating synaptic strength within the buccal oscillator network or modulating some other drive to this oscillator.

Occurrence of ventilatory bursts. Treatment with 7-NI produced a dose-dependent reduction in the occurrence of LV bursts. Thus, although endogenous NO synthesis does not regulate the intrinsic frequency of the oscillator producing this burst pattern, the expression of this oscillator appears to be dependent on the endogenous production of the neuromodulator. This observation is consistent with the lung CRG being a conditional oscillator, having the capacity to oscillate but requiring an excitatory input to induce this oscillation (49). Hedrick and co-workers (19, 20) have also demonstrated that treatment with 7-NI decreases the occurrence of LV in a similar in vitro preparation derived from adult frogs. The authors conclude that NO was somehow necessary for the expression of this discharge and may, thus, be a necessary messenger for neurotransmission and/or modulation of central respi-

Fig. 5. Event-triggered averages (solid line) ± SE (dashed lines) of CN VII activity, demonstrating the influence of various concentrations of 7-NI (in superfusate with pH 7.8) on ventilatory burst shape and pattern. Tracings were triggered from the largest peak of Bessel-filtered CN VII activity and averaged over 25 consecutive episodes of lung burst activity (either the 1st burst of a cluster or a single burst).

Fig. 6. Event-triggered averages (solid line) ± SE (dashed lines) of CN VII activity, demonstrating the influence of various concentrations of 7-NI (in superfusate with pH 8.0) on ventilatory burst shape and pattern. Tracings were triggered from either 1) the largest peak of Bessel-filtered CN VII activity and averaged over 25 consecutive episodes of lung burst activity (either the 1st burst of a cluster or a single burst) or 2) in the absence of lung burst activity, from the characteristic “trough” after a buccal burst and averaged over 25 consecutive 10-s periods.
ratory drive. Our results agree with this conclusion and indicate that NO synthesis influences the expression of LV bursts upstream and/or downstream of the oscillator, rather than by influencing the properties of the oscillator itself. Endogenously produced NO does appear to contribute to tonic excitation of the lung oscillator. Thus this influence facilitates and may be necessary for the expression (occurrence) of LV bursts. Robust BV bursts generally persist while LV bursts become less common as endogenous NO synthesis is disrupted. This suggests that motor output itself is not compromised by 7-NI treatment. An exception, however, may be occurring in the current study at pH 7.6 during exposure to 1.0 mM 7-NI. Under these conditions the expression of both BV and LV bursts appears to be disrupted. A significance to the effect of this relatively high 7-NI dose is unknown.

Shape of ventilatory bursts. In adult and larval frogs, inspiration is initiated by depression of the buccal floor and acquisition of air through open nares into the buccal cavity (constituting buccal acquisition or the loading phase of the buccal force pump). Lung inflation occurs with the elevation of the buccal floor, with closed nares and an open glottis. Expiration immediately precedes lung inflation during tidal ventilation and occurs when the glottis and nares are opened between buccal acquisition and lung inflation (7, 9, 13, 30, 31, 51, 64, 67). Although the removal of peripheral inputs does alter respiratory motor pattern, biphasic LV bursts recorded in vitro correspond to the burst discharge pattern of respiratory muscles during the ventilatory cycle in vivo, with the first and second waves corresponding to activation of buccal depression and elevation, respectively (13, 25, 30, 31, 51).

7-NI treatment abolishes the first element of the biphasic burst in CN VII without any apparent alteration of either the second phase of these bursts or the BV burst (Figs. 5 and 6). This indicates that NO plays an important role in the modulation of LV burst shape, particularly the buccal depression wave or loading phase. The dose-dependent abolishment of the initial phase of normally biphasic LV bursts by NOS inhibition results in a monophasic bursts. This transition suggests that endogenous NO production is essential for the genesis of biphasic ventilatory bursts and may indicate a role of NO in the determination of biphasic (tidal) ventilatory events vs. monophasic (deflationary) events (66).

Despite contributing to a distinct biphasic to monophasic transition in LV burst shape, 7-NI had no effect on the duration of these bursts. The occurrence of what resembled a BV-like rising phase in place of the initial phase of a biphasic burst resulted in a monophasic burst having the same duration as the biphasic bursts that occurred in the absence of NOS inhibition. The significance of such an alteration of burst shape with a preservation of burst duration is unknown.

Clustering bursts into multiburst episodes. During periodic breathing, the tendency to cluster breaths into multibreath episodes is an important element of ventilatory control and specifically regulated by the central nervous system (35, 36). The present results illus-
that NOS inhibition generally reduces the number of putative lung breaths per cluster and prompts the occurrence of single breaths rather than clusters. Such a transition can occur without a reduction in the overall occurrence of breaths, indicating that the regulation of burst clustering can occur independent of overall ventilatory occurrence. Abundant evidence indicates that supramedullary influences contribute to the production of multibreath breathing episodes (23, 27, 39, 40, 42, 43, 46; for reviews, see Refs. 35, 36). The neural elements and mechanistic basis for the production of multibreath episodes are, however, unknown. Our data demonstrate that endogenous NO synthesis is strongly involved in, if not necessary for, clustering breaths into multibreath episodes. Recently Straus et al. (59) have suggested that a pathway dependent on GABA B-type receptors could also regulate such clustering. Given these results, it is possible that several neuromodulators may influence breath clustering.

The site of action for such modulation is uncertain. A number of investigations have suggested that multibreath clusters are the result of specific supramedullary modulatory influences (23, 27, 39, 40, 42, 43, 46; for reviews, see Refs. 35, 36). We routinely observe multiburst clusters of LV activity in isolated brain stem preparations after midtectal transection at the level of the CN III (Ref. 57 and present investigation), but not after isolation of the medulla from the tectum (63). These observations suggest that elements necessary for the generation of multiburst clusters could reside in the tectum caudal to CN III. NOS-containing neurons have been identified in a number of amphibian brain stem regions (2, 5, 14). High concentrations occur in the mid to caudal tectum proximal to the nucleus isthmi, an area involved in the genesis of multibreath clusters as well as modulating ventilatory sensitivity to respiratory stimuli (10, 27). This distribution identifies a potential mechanism through which the isthmic region modulates this clustering. Without discounting a supramedullary influence, we reserve the opinion that the genesis of multibreath breathing episodes may not require a specific, distinct, and necessary neural element such as an episode center. Modulatory inputs of many sorts and sources appear to alter the probability that breaths will occur in clusters (23, 26–28, 35, 36, 59; present investigation). Thus clustered breaths may result from more general properties of the central pattern generator for ventilation itself, rather than from the influence of an episode center per se.

**Sensitivity of In Vitro Ventilatory Pattern to pH/CO₂**

**Specific frequency.** Regardless of pH, the specific frequency of LV bursts within clusters was invariant, suggesting that central chemosensory inputs do not modulate the frequency of the LV oscillator. Although statistically significant, we cannot determine the biological relevance of a BV burst specific frequency higher at pH 7.8 than either 7.6 or 8.0. The presence of this difference, however, suggests that the oscillator responsible for BV burst generation is frequency modulated by chemosensory inputs, while that underlying LV burst generation is not, as we have recently suggested (50). Again, CO₂/pH would appear to influence network properties of the buccal oscillator but not pacemaker properties of the lung oscillator.

**Occurrence.** The occurrence of LV bursts was stimulated by a reduction in pH, confirming the presence of CO₂/H⁺ chemosensitivity within the in vitro brain stem preparation. Thus, although the frequency of the LV oscillator is insensitive to CO₂/H⁺, the expression of this oscillator appears to be conditionally dependent on such input. The degree of this sensitivity is comparable with that previously observed in similar in vitro preparations and in adult frogs in vivo after vagotomy (26, 62). These findings differ from other reports of amphibian in vitro preparations displaying little or no sensitivity to hypercapnia (46, 50). Fewer BV bursts occurred at lower pH. As BV bursts occur rhythmically and relatively continuously in the absence of LV bursts, the reduced occurrence of BV bursts likely reflects the lower opportunity for such bursts to occur under conditions which stimulate LV bursts.

Clustering. LV bursts generally occurred in multiburst clusters, with fewer bursts occurring per cluster at pH 8.0 than at a lower pH. This trend appears to match our finding of a lower LV occurrence rate at higher pH and illustrates a consistent relationship between ventilatory drive (the occurrence of bursts) and the tendency to cluster bursts into multibreath episodes. This distinct influence of pH on LV burst clustering also emphasized the hypercapnic sensitivity of this preparation.

While the specific frequency of LV bursts occurring in episodes is unrelated to pH, we note a marginal influence of pH on the duration of these episodes. In other words, with a constant specific frequency of LV bursts, a tendency toward single breaths at pH 8.0 should also be reflected in a tendency toward shorter episode durations at this pH. The strength of this relationship, however, appears to be minimal.

**Possible role of NO modulation of breathing pattern.** The core of the tadpole in vitro brain stem preparation is hyperoxic and mildly acidic, resulting from a respiratory acidosis and restricted CO₂/H⁺ diffusion from the nonperfused tissues (61, 69). Undoubtedly, LV burst activity in these preparations is sensitive to PCO₂/pH and will be facilitated by the core tissue acidosis. Yet, in the absence of other peripheral inputs, stimulation from these levels of CO₂/H⁺ in vivo may not result in such robust respiratory patterns (28, 55, 56). As NO synthesis is enhanced by hyperoxia (1), we agree with the contention of Hedrick and Morales (19) that tissue NO may be elevated and that this elevated NO stimulates the production of LV bursts.

The PO₂ of our hyperoxic in vitro preparations is within the range (0–150 Torr) over which NOS activity is exquisitely sensitive to O₂ (1). We propose that tissue PO₂ normally modulates NOS activity over this range and, thus, regulates endogenous NO synthesis. Hypoxia could then result in a reduction of tissue NO,
which is of primary importance to the modulation of a number of physiological factors that underscore such phenomena as hypoxic ventilatory and metabolic depression (12, 38). Recently, Gargaglioni and Branco (10) have reported that disrupting NO synthesis within the nucleus isthmi of toads enhances their ventilatory responses to hypoxia or hypercarbia. These results indicate that reductions in NO synthesis within specific brain stem areas can augment ventilatory sensitivity to respiratory stimuli and suggest that NO synthesis could be involved in the transduction or integration of chemosensory information. We further postulate that endogenous tissue NO synthesis provides central oxygen sensitivity to some if not all neural tissues, allowing such tissues to arrest their function and metabolism in the face of hypoxia, thereby shielding themselves from hypoxic damage. The NOS enzyme is found in organisms ranging from bacteria to vertebrates and in most energetically active tissues prone to damage by hypoxia (21, 41). Given the relatively universal nature of NO and its potent role as a modulator, a mechanism of NO-mediated hypoxia-induced hypometabolism may be highly significant, and both ancient and highly conserved.

Oscillatory mechanisms for BV and LV. Amphibians exhibit BV and LV oscillations, two rhythmic patterns that could result from the same or distinct neuronal oscillators (28). In the present investigation, the specific frequency of LV bursts within a cluster was constant regardless of pH or the concentration of 7-NI. In contrast, the specific frequency of BV bursts 1) was lower than that of LV bursts under initial conditions, 2) was influenced by pH, and 3) increased with 7-NI treatment. The oscillator generating BV bursts, therefore, appears to have a different endogenous period and be differentially modulated than that which generates LV bursts. These differences lend further support to the hypothesis that LV and BV bursts are generated by functionally distinct oscillators.

Conclusion

Endogenous NO synthesis has a diverse influence on respiratory burst activity. It modulates the period of a BV oscillator and the conditional expression of a functionally distinct LV oscillator. It influences the shape, and perhaps function, of LV bursts and the tendency for such bursts to cluster into multiburst episodes. These influences could explain the presence of robust ventilatory rhythms in hyperoxic in vitro preparations that lack other modulatory inputs, and they may be highly significant in mediating adaptive responses to hypoxia in vivo.

We thank M. Hedrick for assisting on the formulation of this investigation.

Research was funded by an operating grant to J. E. Remmers from the Medical Research Council of Canada and by fellowships from the Alberta Heritage Foundation for Medical Research (R. J. A. Wilson and K. Vasilakos), the Medical Research Council of Canada (K. Vasilakos), the Natural Science and Engineering Research Council of Canada (M. B. Harris), and the Parker B. Francis Foundation for Pulmonary Research (R. J. A. Wilson).

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