Patterns of Phrenic Motor Output Evoked by Chemical Stimulation of Neurons Located in the Pre-Bötzing Complex In Vivo

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Soledom, Irene C., Norman H. Edelman, and Judith A. Neubauer. Patterns of phrenic motor output evoked by chemical stimulation of neurons located in the pre-Bötzing complex in vivo. J. Neurophysiol. 81: 1150–1161, 1999. The pre-Bötzing complex (pre-Bo¨tC) has been proposed to be essential for respiratory rhythm generation from work in vitro. Much less, however, is known about its role in the generation and modulation of respiratory rhythm in vivo. Therefore we examined whether chemical stimulation of the in vivo pre-Bo¨tC manifests respiratory modulation consistent with a respiratory rhythm generator. In chloralose- or chloralose/urethan-anesthetized, vagotomized cats, we recorded phrenic nerve discharge and arterial blood pressure in response to chemical stimulation of neurons located in the pre-Bo¨tC with α-homocysteic acid (DLH; 10 mM; 21 nl). In 115 of the 122 sites examined in the pre-Bo¨tC, unilateral microinjection of DLH produced an increase in phrenic nerve discharge that was characterized by one of the following changes in cycle timing and pattern: 1) a rapid series of high-amplitude, rapid rate of rise, short-duration bursts, 2) tonic excitation (with or without respiratory oscillations), 3) an integration of the first two types of responses (i.e., tonic excitation with high-amplitude, short-duration bursts superimposed), or 4) augmented bursts in the phrenic neurogram (i.e., eupneic breath ending with a high-amplitude, short-duration burst). In 107 of these sites, the phrenic neurogram response was accompanied by an increase or decrease (±10 mmHg) in arterial blood pressure. Thus increases in respiratory burst frequency and production of tonic discharge of inspiratory output, both of which have been seen in vitro, as well as modulation of burst pattern can be produced by local perturbations of excitatory amino acid neurotransmission in the pre-Bo¨tC in vivo. These findings are consistent with the proposed role of this region as the locus for respiratory rhythm generation.

INTRODUCTION

A region of the ventrolateral medulla is postulated to be essential for respiratory rhythm generation (for a recent review see Reckling and Feldman 1998). This region has been termed the pre-Bötzing complex (pre-Bo¨tC), and it is located between the rostral ventral respiratory group (rVRG) and the Bötzing (Bo¨t) complex (Feldman et al. 1990; Smith et al. 1991). Evidence identifying a unique role for the pre-Bo¨tC in respiratory rhythm generation comes primarily from work on in vitro neonatal rodent brain stem–spinal cord preparation (Smith et al. 1991), and in vitro medullary slices that contain this region continue to generate respiratory oscillations in cranial motoneurons (Funk et al. 1994; Smith et al. 1991).

An analogous region has been identified in the adult cat based on both its electrophysiological characteristics and anatomic boundaries (Connelly et al. 1992; Ramirez et al. 1998; Schwarzacher et al. 1995). In the adult cat, the pre-Bo¨tC is characterized by a mix of neurons with inspiratory-modulated, expiratory-modulated, and phase-spanning patterns of discharge (Connelly et al. 1992; Schwarzacher et al. 1995). Compared with the adjacent rVRG and Bo¨t complex, there appears to be a high-density of phase-spanning neurons that exhibit preinspiratory (pre-I) discharge patterns. As in the rat, the rostral pole of the pre-Bo¨tC lies immediately caudal to the retrofacial nucleus (Connelly et al. 1992; Ramirez et al. 1998; Schwarzacher et al. 1995), in a region ventrolateral to the compact formation of nucleus ambiguus. In the rostrocaudal plane, it has been identified as lying rostral to the rostral pole of the lateral reticular nucleus and to be located at about the halfway point of the inferior olivary nucleus and the rostral pole of the hypoglossal nucleus (Ramirez et al. 1998; Schwarzacher et al. 1995).

Although the role of pre-Bo¨tC has received much attention in the in vitro brain stem–spinal cord and medullary slice preparations, much less is known about its role in the generation and modulation of respiratory rhythm in vivo (Abrahams et al. 1991; Connelly et al. 1992; Ramirez et al. 1998; Schwarzacher et al. 1995). If this region is responsible for generation of respiratory rhythm, we predict that stimulation of neurons located in this region will increase the frequency and alter the patterning of inspiratory motor output, in a manner unique to this region. Additionally, because the central respiratory rhythm generator exerts an influence on sympathetic output, we predict that activation of neurons located in the pre-Bo¨tC will also modify blood pressure.

METHODS

General

Experiments were conducted in 49 adult cats, weighing 2.8–5.0 kg. Twenty-one cats were anesthetized initially with ketamine hydrochloride (15 mg/kg im) and acepromazine maleate (1.1 mg/kg im). The right brachial vein was cannulated and α-chloralose (10–20 mg/kg iv) was administered intravenously. Supplemental α-chloralose was
given as needed (3–5 mg/kg iv). The remaining 28 cats were anesthetized initially by placing them inside a sealed, plastic induction chamber into which a gaseous mixture of halothane (5%) and oxygen was introduced. After the cats were anesthetized, they were removed from the chamber, and anesthesia was maintained by delivering halothane (1.5–3%) and oxygen through a face mask placed over the cat’s nose and mouth. The right brachial vein was cannulated, and α-chloralose and urethan (35 and 210 mg/kg iv, respectively) were administered. The gaseous anesthesia was then removed. Supplemental α-chloralose and urethan (5 and 10 mg/kg iv, respectively) were given as needed. The adequacy of anesthesia was regularly verified by firmly pinching a toe. If an increase in blood pressure was evoked or, during the absence of paralysis (see Experimental protocol), if the cat withdrew its limb, additional anesthesia was given. In all cats, both brachial arteries were cannulated. The left brachial cannula was connected to a Statham transducer (P23Db) for measurement of arterial blood pressure; the right brachial cannula was used for sampling arterial blood.

The trachea was cannulated low in the neck and the lungs mechanically ventilated with room air enriched with O₂. The chest was opened bilaterally through the sixth intercostal spaces, and the expiratory outlet of the ventilator was placed under 1–2 cm H₂O to prevent opening bilaterally through the sixth intercostal spaces, and the expiratory outlet of the ventilator was placed under 1–2 cm H₂O to prevent collapse of the lungs during expiration. End-tidal CO₂ was monitored continuously through a side port in the tracheal cannula and maintained between 4.0 and 5.5% by adjusting the tidal volume and respiratory frequency of the ventilator. Arterial PO₂, P CO₂, and pH were measured at hourly intervals (Radiometer ABL-30), and when necessary, blood gas values were corrected by either adjusting the ventilator or intravenous infusion of sodium bicarbonate (8.5%). At the start of the experimental protocol, arterial P CO₂, PO₂, and pH averaged 225 ± 6 (SE) mmHg, 39 ± 1 mmHg, and 7.40 ± 0.01, respectively. Body temperature was measured and maintained at 36–38°C with the use of a heating pad and a heat lamp.

Both cervical vagus nerves were exposed and cut bilaterally. In 25 cats, the carotid sinus nerves were also exposed and cut bilaterally. The cat’s head was then placed in a stereotoxic instrument and the dorsal surface of the medulla exposed by separating the nuchal musculature along the midline, removing the basioccipital bone, and opening the atlantooccipital membrane. The dorsal surface of the brain stem was covered with warm mineral oil to prevent drying.

The C₅ rootlet of one or both phrenic nerves was isolated in the neck via a lateral approach, cut, desheathed, and the central end was placed on a bipolar hook electrode. The nerve was then covered with a mixture of mineral oil and petroleum jelly. Phrenic nerve discharge was amplified (×1000–10,000) and filtered (1–10,000 Hz), and a moving average was obtained using a third-order Paynter filter with a 100-ms time constant. Both the raw and averaged nerve outputs were recorded on digital tape (A. R. Vetter, Model 4000A) and on a chart recorder.

**Experimental protocol**

We examined the effects of chemical stimulation of neurons located in the pre-Bo¨tC with dl-homocysteic acid (DLH), a glutamate analogue, on phrenic nerve discharge and arterial blood pressure. Responses from a total of 122 sites in the pre-Bo¨tC were recorded. Responses from an additional 30 sites adjacent (within 200–500 μm) to the pre-Bo¨tC, including the rVRG and the Bö complex, were also recorded. All sites were initially localized using predetermined stereotoxic coordinates relative to the calamus scriptorius (3.4–4.0 mm rostral), midline (3.8–4.2 mm lateral), and dorsal surface (4.2–4.5 mm ventral) and histologically confirmed (see Location of responsive and nonresponsive sites). The range in values for the coordinates used to find the pre-Bo¨tC reflect the variability in the dimensions of the brain stems in cats of different size.

All microinjections into the medulla were made with the use of a triple-barreled glass pipette (20–40 μm tip diameter) attached to a pressure injection device (General Valve Picospritzer II). One barrel of the pipette contained 10 mM DLH. The second barrel contained saline. The third barrel contained Fast Green dye (2%), which was used to mark the injection sites (≤105 nl). All microinjected chemicals were dissolved in a saline solution, and the pH was adjusted to 7.36–7.42. Microinjection volumes of DLH and saline were 21 nl, and microinjection typically required 1–2 s to complete. The volume of each injection was monitored by observing the displacement of the fluid meniscus using a microscope equipped with an eyepiece reticule. To control for nonspecific effects, equivalent volumes of saline were microinjected into sites in which DLH elicited ventilatory or cardiovascular changes.

In 27 of the 49 cats, we conducted a series of mapping experiments in the pre-Bo¨tC and adjacent areas in response to microinjection of DLH. No more than four microinjections (including those into adjacent areas) were made on one side of the brain stem, and bilateral microinjections were made in most animals. In general, our first site in these experiments was determined using the stereotoxic coordinates defined above, with subsequent microinjections made after movement of the pipette in 200-to 300-μm increments in the rostral-caudal, medial-lateral, or dorsal-ventral direction. Most often, the pipette was moved in a rostral-caudal direction. In the remaining 22 cats, a total of 1–3 sites were examined in the pre-Bo¨tC.

Baseline phrenic neurogram amplitude was initially set at 40–60% of the maximum amplitude evoked by rebreathing CO₂ in O₂ until end-tidal CO₂ increased to 8.0%. Thus we were able to record increases or decreases in phrenic neurogram amplitude evoked by microinjection of DLH into the medulla. Before stimulation of sites in the medulla, the cats were paralyzed with gallamine triethiodide (2 mg/kg iv). The effect of the paralytic agent was periodically allowed to dissipate so that the level of anesthesia could be assessed. Supplemental doses of the paralytic agent were administered as needed.

**Histology**

At the conclusion of the experiment, the cat was killed under deep anesthesia by an injection of saturated KCl solution. The brain stem was removed and placed in 4% Formalin for at least 48 h. The brain stem was then frozen, sectioned coronally (40 μm), mounted on slides, and stained for cell bodies with the use of 1% Neutral Red dye. With the use of a microprojector, we made drawings of tissue sections containing sites marked with Fast Green dye.

**Data analysis**

Amplitude of integrated phrenic nerve discharge, inspiratory time (T₁), expiratory time (Tₑ), and rate of rise were determined from the phrenic neurogram off-line (AT CODAS; DATAQ Instruments). Amplitude of integrated phrenic nerve discharge is reported as a percentage of the maximal amplitude recorded in each cat. This maximal amplitude occurred either in response to ventilating the lungs with CO₂ (see Experimental protocol) or in response to microinjection of DLH into the medulla. Rate of rise was determined over the linear phase of activity and is reported as a percentage of the maximal rate of rise recorded in each cat. Baseline values for all of these variables were determined by averaging the values obtained for the 60-s period immediately preceding microinjection of DLH into the medulla. Peak response values for these variables were determined by averaging the first five consecutive breathing cycles that displayed the greatest change from baseline values. Baseline values for mean arterial pressure were taken as the steady-state values. Peak responses were taken as the highest or lowest value reached after microinjection of DLH. All values are reported as means ± SE. Responses before and after stimulation are presented as paired data. A Student’s paired t-test or ANOVA was used to determine statistical significance, for which the criterion level was set at P < 0.05.
## RESULTS

**General effects of microinjection of DLH into the pre-BötC**

Unilateral microinjection of DLH (10 mM, 21 nl) into discrete sites in the pre-BötC produced an increase in phrenic nerve discharge in 115 of the 122 sites examined. This augmentation of inspiratory output exhibited one of the following changes in cycle timing and pattern: 1) a rapid series of high-amplitude, short-duration bursts, 2) tonic excitation (with or without respiratory oscillations), 3) a mixture of the first two types of responses (i.e., tonic excitation with high-amplitude, short-duration bursts superimposed), or 4) augmented bursts in the phrenic neurogram (i.e., eupneic breath ending with a high-amplitude, short-duration burst). These responses will be described in more detail below. In 66 sites, the phrenic neurogram response was accompanied by a marked (≥30 mmHg) increase or decrease in arterial blood pressure; however, changes in arterial blood pressure (≥10 mmHg) accompanied phrenic neurogram responses in 107 of the sites examined. In general, the onset of the respiratory effects preceded the blood pressure response.

Responses to microinjection of DLH were reproducible following recovery to a eupneic pattern. In general, we waited at least 10 min before attempting to demonstrate repeatability. Microinjections of equivalent volumes of saline into responsive sites were ineffective in producing any changes in phrenic nerve discharge or blood pressure. Additionally, we encountered seven sites in the pre-BötC in which microinjection of DLH failed to induce any effect on phrenic nerve discharge or blood pressure.

Similar phrenic neurogram and blood pressure responses were observed in cats under both anesthetic paradigms. Additionally, similar phrenic neurogram and blood pressure responses were observed in cats with carotid sinus nerves intact or cut. Therefore these data will not be considered separately.

**Rapid series of high-amplitude, short-duration bursts**

Unilateral microinjection of DLH into 31 sites in the pre-BötC produced a rapid series of high-amplitude, rapid rate of rise, short-duration bursts in the phrenic neurogram (Fig. 1). This response typically occurred within 1–2 s from the beginning of microinjection and had durations ranging from 15 to 172 s. Bilateral recordings were obtained in response to microinjection of DLH into 14 of these 31 sites in the pre-BötC. The effects on phrenic nerve discharge were bilaterally symmetrical in those animals with bilateral recordings. On average, microinjection of DLH into these sites decreased $T_i$ from $1.57 ± 0.14$ to $0.34 ± 0.02$ s ($P < 0.01$), decreased $T_e$ from $2.32 ± 0.27$ to $0.64 ± 0.12$ s ($P < 0.01$), increased the amplitude of integrated phrenic nerve discharge from $30.8 ± 5.1$ to $85.6 ± 2.5\%$ ($P < 0.01$) of maximum, and increased the rate of rise of phrenic nerve activity from $6.4 ± 1.1$ to $79.4 ± 4.3\%$ ($P < 0.01$) of the maximal rate of rise (Fig. 2).

In 22 of these sites, this rapid series of high-amplitude, short-duration bursts in the phrenic neurogram was accompanied by a marked increase in mean arterial pressure from $107 ± 6$ to $148 ± 8$ mmHg ($P < 0.01$). In the remaining nine sites, the rapid series of bursts was accompanied by a decrease in mean arterial pressure from $100 ± 14$ to $68 ± 14$ mmHg ($P < 0.01$).

**Tonic excitation**

Unilateral microinjection of DLH into 79 sites in the pre-BötC produced a tonic excitation of phrenic nerve discharge. This tonic excitation may be grouped into three types of responses: 1) nonrhythmic excitation, 2) rhythmic excitation, and 3) tonic excitation with high-amplitude, short-duration bursts superimposed (see *Tonic excitation with high-amplitude, short-duration bursts superimposed*). Bilateral recordings were obtained in response to microinjection of DLH into 56 of these 79 sites in the pre-BötC. The effects on phrenic nerve discharge were bilaterally symmetrical in those animals with bilateral recordings.

Nonrhythmic excitatory responses were evoked in 46 of these 79 sites and were characterized by an abrupt rise in phrenic nerve discharge to a plateau level (Fig. 3). In most cases, the peak amplitude of integrated phrenic nerve discharge...
at the onset of the response was higher or the same as that seen during baseline breaths; however, in a few cases, peak amplitude was slightly reduced. At the onset of the response, an increase in the rate of rise of phrenic activity was usually present. The response typically occurred within 1–4 s from the beginning of microinjection and had durations ranging from 27 to 434 s. This type of tonic excitation exhibited a gradual recovery (n = 28) or was followed by a transient postexcitatory depression of phrenic nerve discharge that lasted 50–516 s (n = 18). In 14 of these sites, the nonrhythmic excitation of phrenic nerve discharge was accompanied by a marked increase in mean arterial pressure from 104 ± 8 to 148 ± 9 mmHg (P < 0.01). In 29 of these sites, the nonrhythmic excitation of phrenic nerve discharge was accompanied by a decrease in mean arterial pressure from 111 ± 6 to 68 ± 5 mmHg (P < 0.01). No change in mean arterial pressure was seen in the remaining three of these sites.

Rhythmic excitatory responses were evoked in 15 of these 79 sites and were characterized by the presence of rhythmic respiratory oscillations superimposed on variable levels of tonic discharge (Fig. 4). In most cases, there was an increase in frequency and/or peak amplitude of phrenic nerve discharge.
The response typically occurred within 1–5 s from the beginning of microinjection and had durations ranging from 21 to 213 s. In addition, this type of tonic excitation exhibited a gradual recovery of phrenic nerve discharge. In four of these sites, the rhythmic excitation of phrenic nerve discharge was accompanied by an increase in mean arterial pressure from $114 \pm 9$ to $136 \pm 6$ mmHg ($P < 0.05$), whereas in the remaining 11 sites, the rhythmic excitation of phrenic nerve discharge was accompanied by a marked decrease in mean arterial pressure from $107 \pm 7$ to $61 \pm 6$ mmHg ($P < 0.01$).

In 6 of the 79 sites, tonic excitation of phrenic nerve discharge changed from a rhythmic excitatory response into a nonrhythmic excitatory response (Fig. 5), and in 1 of the 79 sites, tonic excitation of phrenic nerve discharge changed from a nonrhythmic excitatory response into a rhythmic excitatory response. These responses typically occurred within 1–5 s from the beginning of microinjection and had durations ranging from 80 to 240 s. In all seven of these sites, the excitation of phrenic nerve discharge was accompanied by a marked decrease in mean arterial pressure from $120 \pm 17$ to $69 \pm 15$ mmHg ($P < 0.01$).

**Tonic excitation with high-amplitude, short-duration bursts superimposed**

Unilateral microinjection of DLH into the remaining 11 of these 79 sites in the pre-BötC produced a tonic excitation of phrenic neurogram output with high-amplitude, short-duration bursts superimposed (Fig. 6). This response was characterized by a rapid series of high-amplitude, short-duration bursts superimposed on a nonrhythmic excitatory response. The onset of the tonic excitation occurred within 1–5 s from the beginning of microinjection and had durations ranging from 21 to 410 s. The superimposed high-amplitude, short-duration bursts began after the onset of the tonic response and lasted only 18–140 s. For these high-amplitude, short-duration bursts, $T_i$ was decreased from $1.42 \pm 0.16$ to $0.30 \pm 0.04$ s ($P < 0.01$), and $T_e$ was decreased from $2.21 \pm 0.32$ to $0.46 \pm 0.08$ s ($P < 0.01$). In addition, the peak amplitude of integrated phrenic nerve discharge and the rate of rise of phrenic nerve activity were increased above baseline values.

In seven of these sites, the phrenic neurogram response was accompanied by a marked increase in mean arterial pressure from $110 \pm 9$ to $157 \pm 13$ mmHg ($P < 0.01$), whereas in the remaining four sites, the phrenic neurogram response was accompanied by a decrease in mean arterial pressure from $124 \pm 23$ to $85 \pm 28$ mmHg ($P < 0.05$).

**Augmented bursts**

Unilateral microinjection of DLH into five sites in the pre-BötC produced augmented bursts in the phrenic neurogram (i.e., eupneic breaths ending with a high-amplitude, short-duration burst). The effects on phrenic nerve discharge were bilaterally symmetrical in all sites examined. These augmented bursts appeared as either a series of augmented breaths (Fig. 7) or were interspersed between eupneic breaths. In some cases, the peak amplitude of integrated phrenic nerve discharge of the eupneic portion of the augmented burst was increased above that seen during baseline breaths. The onset of augmented
breaths occurred within 1–5 s from the beginning of microinjection and had durations ranging from 44 to 208 s. In these sites, the phrenic neurogram response was accompanied by small increases or decreases in mean arterial pressure.

Microinjection of DLH into sites adjacent to the pre-Bötzinger complex

We also examined the effects of unilateral microinjection of DLH into 30 sites adjacent to the pre-Bötzinger complex as a control for the spread of the injectate. These microinjections were made into sites in the rVRG, Bötz complex, and 200–500 μm dorsomedial, dorsolateral, or lateral to the pre-Bötzinger complex.

In 11 of 12 sites in the rVRG, microinjection of DLH produced site-specific increases (n = 2) or decreases (n = 9) in peak amplitude of integrated phrenic nerve discharge with little or no change in cycle timing or frequency of bursts (Fig. 8A). In 4 cases, the decrease in peak amplitude of integrated phrenic nerve discharge led to phrenic apnea. Responses evoked by microinjection of DLH into the rVRG had an onset latency of 2–3 s from the beginning of microinjection and had durations ranging from 40 to 98 s. In 6 of these 11 sites, the phrenic neurogram response was accompanied by a marked decrease in mean arterial pressure from 116 ± 20 to 83 ± 26 mmHg (P < 0.05). In the remaining 5 of these 11 sites, the phrenic neurogram response was accompanied by a small

FIG. 5. Example of an excitatory response that changes from rhythmic to nonrhythmic in the phrenic neurogram evoked by microinjection of DLH (10 mM; 21 nl) into the pre-Bötzinger complex. Traces from top to bottom: BP, integrated phrenic nerve activity (ipsilateral), and raw phrenic nerve activity (ipsilateral), integrated phrenic nerve activity (contralateral), and raw phrenic nerve activity (contralateral). Note also that microinjection of DLH into this site evoked a decrease in arterial blood. Trace on right (Recovery) shows full recovery of BP and phrenic nerve activity at ~20 min after microinjection of DLH.

FIG. 6. Example of a tonic excitatory response in the phrenic neurogram with a rapid series of high-amplitude, short-duration bursts superimposed evoked by microinjection of DLH (10 mM; 21 nl) into the pre-Bötzinger complex. Traces from top to bottom: BP, integrated phrenic nerve activity (contralateral), and raw phrenic nerve activity (contralateral). Note also that microinjection of DLH into this site evoked an increase in arterial blood pressure.
increase or no change in mean arterial pressure. Microinjection of DLH into the remaining site in the rVRG failed to induce any effect on phrenic nerve discharge or blood pressure.

In six sites in the Böt complex, microinjection of DLH produced phrenic apnea (Fig. 8B). In one of these sites, apnea was preceded by a decrease in peak amplitude of integrated

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**FIG. 7.** Example of a series of augmented bursts in the phrenic neurogram evoked by microinjection of DLH (10 mM; 21 nl) into the pre-BötC. Traces from top to bottom: BP, integrated phrenic nerve activity (contralateral), and raw phrenic nerve activity (contralateral), integrated phrenic nerve activity (ipsilateral), and raw phrenic nerve activity (ipsilateral). Note also that microinjection of DLH into this site evoked no change in arterial blood pressure.

**FIG. 8.** A: example of an increase in peak amplitude of phrenic nerve discharge evoked by microinjection of DLH (10 mM; 21 nl) into the rostral ventral respiratory group (rVRG). Traces from top to bottom: BP, integrated phrenic nerve activity (ipsilateral), and raw phrenic nerve activity (ipsilateral). Note also that microinjection of DLH into this site evoked an increase in arterial blood pressure. B: example of phrenic apnea evoked by microinjection of DLH (10 mM; 21 nl) into the Böt complex. Traces from top to bottom: BP, integrated phrenic nerve activity (contralateral), and raw phrenic nerve activity (contralateral). Note also that microinjection of DLH into this site evoked a decrease in arterial blood pressure.
burst superimposed on a tonic discharge. In fact, produced augmented bursts and high-amplitude, short-duration within this region are the sites in which microinjection of DLH short-duration bursts in a fairly restricted region. Also included produced a rapid series of high-amplitude, rapid rate of rise, and in the remaining two sites, the phrenic neurogram response was not accompanied by a change in mean arterial pressure.

In 12 sites located dorsomedial, dorsolateral, or lateral to the pre-Boëtzinger, microinjection of DLH elicited no effect on phrenic nerve discharge. In one of these sites, a small decrease in mean arterial blood pressure was seen.

**Location of responsive and nonresponsive sites**

The distribution of sites in which DLH was microinjected into the medulla is shown in Fig. 9. As landmarks for identifying the rostrocaudal level of the pre-Boëtzinger, we identified the caudal pole of the retrofacial nucleus, nucleus ambiguus, the rostral pole of the lateral reticular nucleus, and the rostral pole of the hypoglossal nucleus. Examining our data from the level of obex produced considerable overlap in the types of responses seen, and placed >90% of our sites in the same coronal section. Therefore, in our analyses, all sites in the pre-Boëtzinger were identified with reference to the caudal pole of the retrofacial nucleus, not the obex. We found that mapping the sites in this way was consistent with the difference in the types of responses obtained based on movement of the pipette using stereotaxic coordinates in our experiments.

As can be noted, microinjection of DLH into the pre-Boëtzinger produced a rapid series of high-amplitude, rapid rate of rise, short-duration bursts in a fairly restricted region. Also included within this region are the sites in which microinjection of DLH produced augmented bursts and high-amplitude, short-duration bursts superimposed on a tonic discharge. In fact, ~87% of the sites that included a high-amplitude, short-duration burst (i.e., rapid series of high-amplitude, rapid rate of rise, short-duration bursts; high-amplitude, short-duration bursts superimposed on a tonic discharge; augmented bursts) were located in this region of the pre-Boëtzinger. In contrast, microinjection of DLH into the pre-Boëtzinger produced a tonic excitation of phrenic neurogram output in a much larger portion of the pre-Boëtzinger. We could not distinguish nonrhythmic from rhythmic excitatory responses based on their histological distribution. Further, we could not differentiate pressor and depressor responses based on their histological distribution.

We were unable to recover tissue sections corresponding to seven sites in the pre-Boëtzinger marked with Fast Green dye. In these cases, we have identified these sites based on either tract markings from the pipette or on their stereotaxic location from adjacent sites that were recovered. In three of these seven sites, microinjection of DLH failed to induce any effect on phrenic nerve discharge or blood pressure. In the remaining four sites, microinjection of DLH produced a tonic excitation of phrenic neurogram output.

**DISCUSSION**

We have demonstrated that chemical stimulation of discrete regions of the pre-Boëtzinger with the glutamate analogue DLH markedly alters both respiratory rhythm and pattern in an excitatory fashion. Further, we have shown that chemical stimulation of neurons located in the rVRG produces site-specific transient increases or decreases in the amplitude of integrated phrenic nerve discharge (McCrimmon et al. 1986), and that chemical stimulation of sites in the Boëtz complex exerted a strong depressant effect on inspiratory motor output (i.e., apnea) (Bongianni et al. 1988). Taken together, our findings suggest that the responses evoked by chemical stimulation of neurons located in the pre-Boëtzinger are unique to this region of the medulla and are consistent with the proposed role of this region as a locus for respiratory rhythm generation.

**Limitations of pressure injection and chemical stimulation**

The excitatory amino acid DLH activates both N-methyl-D-aspartate (NMDA) and non-NMDA [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate] excitatory amino acid receptors that are located on dendrites and cell bodies, but not fibers of passage (Engberg et al. 1979). Because excitatory amino acid receptors are located on most neurons within the CNS, DLH stimulation may have depolarized neurons not normally activated in synchrony or may have increased rates of firing of individual neurons beyond the normal range. Acknowledging these limitations, we believe that in these experiments the transient bilateral increases in phrenic nerve discharge evoked by unilateral microinjection of DLH into the pre-Boëtzinger are highly likely to have resulted from chemical activation of neurons in the immediate vicinity of the pipette tip (Goodchild et al. 1982). It is unlikely that these responses resulted from nonspecific effects of pressure of the injectate, because they were not produced by equal volumes of saline or larger volumes of Fast Green dye from adjacent barrels of the same pipette. Further, although Lipski et al. (1988) suggest that pressure injections of excitatory amino acids into the CNS can produce depolarization block of neurons following the initial neuronal excitation, this too is unlikely because our doses of DLH were relatively small (10 mM; 21 nl) compared with those used by Lipski et al. (0.5–1.0 M; 10–150 nl), our responses were reproducible with repeated injections (even before recovery to eupnea), and DLH is less likely than glutamate to produce depolarization block (Engberg et al. 1979).

The effective spread of injectate is difficult to gauge without direct measurement. Nevertheless, the dose of DLH used in our experiments was relatively small and probably did not spread far from the injection site. Theoretical calculations by Nicholson (1985) demonstrate that based on a 10-nl injection into brain tissue, the concentration of injectate 300 μM from the injection site never exceeds 20% of the initial injectate concentration. Additionally, Lipski et al. (1988) estimated that microinjection of a 30-nl volume in a dose ~100 times larger than that used in our experiments had a radius spread of 325 μm. This estimation appears to be consistent with our finding that movement of the pipette tip by as little as 200 μm produced marked differences in the phrenic neurogram responses. We cannot exclude, however, the possibility that

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FIG. 9. Schematic drawings of coronal sections of the medulla showing sites in which microinjections of DLH were made. All sites in the pre-BoëC are identified with reference to the caudal pole of the retrofacial nucleus (0 mm). Each section is meant to encompass level indicated ±0.2 mm (rostrally and caudally). A: sites in which microinjection of DLH evoked high-amplitude, short-duration bursts (● and ○). B: sites in which microinjection of DLH evoked nonrhythmic excitatory responses (● and ○), rhythmic excitatory responses (■ and □), and tonic excitatory responses that changed from rhythmic to nonrhythmic or from nonrhythmic to rhythmic (▲ and Δ). C: sites in which microinjection of DLH evoked tonic excitatory responses with high-amplitude, short-duration bursts superimposed (● and ○) and augmented bursts (■ and □). D: sites adjacent to the pre-BoëC (rVRG, ● and ○; Boë complex, ■ and □) and nonresponsive sites in and adjacent to the pre-BoëC (▲). A–D: ●, ■, and ▲, sites at which respiratory responses were accompanied by increases in arterial blood pressure; ○, □, and Δ, sites at which respiratory responses were accompanied by decreases in arterial blood pressure; hatched symbols, sites at which respiratory responses were not accompanied by changes in arterial blood pressure. NA, nucleus ambiguous; RFN, retrofacial nucleus; LRN, lateral reticular nucleus; 5SP, nucleus of the trigeminal nerve; 5ST, tract of the trigeminal nerve; and ION, inferior olivary nucleus.
Effects of chemical stimulation in the pre-BötzC on respiratory output

It is unlikely that our DLH-induced effects resulted from direct activation of phrenic premotor neurons. Synchronous antidromic activation of bulbospinal inspiratory neurons does not elicit resetting of the respiratory rhythm, suggesting that these neurons play a limited role, if any, in respiratory rhythm generation (Feldman et al. 1984). Further, anatomic studies in adult rats have demonstrated that the pre-BötzC contains a high concentration of respiratory interneurons with very few premotor neurons compared with adjacent regions (Ellenberger and Feldman 1990; Smith et al. 1991). This relative lack of bulbospinal neurons from the anatomic studies has been confirmed electrophysiologically in adult cats (Schwarzacher et al. 1995). Because microinjection of DLH into the pre-BötzC produces phase resetting of the respiratory rhythm (indicated by our increases in respiratory burst frequency), our findings are most consistent with stimulation of a respiratory rhythm generator. We believe that chemical stimulation of neurons located in this region directly affects the neurons involved in generation of respiratory rhythm because this region is analogous to that in the rat, which has been demonstrated to represent a primary locus of respiratory rhythm generation (Smith et al. 1991).

Feldman and Smith and colleagues have proposed a hybrid model for respiratory rhythm generation whereby, at least in the neonate, voltage-dependent pacemaker cells in the pre-BötzC provide rhythmic drive to the rest of the respiratory network during the inspiratory phase of network activity (Feldman and Smith 1995; Reckling and Feldman 1998; Smith et al. 1991, 1995). In their model, synaptic interactions synchronize and modify the basic rhythm generator by affecting the intrinsic membrane conductances of these voltage-dependent pacemaker neurons. These pacemaker neurons, therefore, have multiple functional states ranging from quiescence (hyperpolarized levels) to oscillatory bursting to tonic action-potential generation (beating) (Smith et al. 1995). Based on the conditional bursting properties of these neurons in the pre-BötzC, our results may be interpreted as follows. In some of our sites in the pre-BötzC, activation of excitatory amino acid receptors by DLH depolarized quiescent pacemaker neurons to a bursting oscillatory state, which resulted in an increase of respiratory burst frequency (i.e., a rapid series of high-amplitude, short-duration bursts). In other sites in the pre-BötzC, activation of excitatory amino acid receptors by DLH depolarized either quiescent or bursting pacemaker neurons to a tonic state of action-potential generation, which resulted in a tonic excitation of inspiratory output. Finally, in sites in which activation of excitatory amino acid receptors by DLH produced both frequency and tonic excitatory effects on inspiratory output, we suggest that pacemaker neurons exhibiting bursting oscillatory behavior and tonic action-potential generation coexist.

Although our findings are consistent with the hybrid model for respiratory rhythm generation proposed by Feldman and Smith and colleagues (Feldman and Smith 1995; Reckling and Feldman 1998; Smith et al. 1991, 1995), they are also compatible with network models of respiratory rhythm generation that do not include conditional pacemaker neurons. Most network models rely on respiratory phase-dependent synaptic interactions among the various types of respiratory-modulated neurons (Balis et al. 1994; Ogilvie et al. 1992; Richter et al. 1986). Changes in the strength of these synaptic interactions and/or changes in activity of a population of respiratory neurons can modify respiratory rhythm and pattern (Balis et al. 1994; Ogilvie et al. 1992). The pre-BötzC contains a high concentration of pre-I neurons (Connelly et al. 1992; Schwarzacher et al. 1995), which appear to be necessary for phase transition (Schwarzacher et al. 1995; Smith et al. 1990). Pre-I neurons are proposed to provide excitatory drive to inspiratory neurons exhibiting both augmenting (I-AUG) and decrementing (I-DEC) patterns of discharge (Segers et al. 1987). In computer simulations inferred from spike train cross-correlation data (Balis et al. 1994) and the mathematical model of a network oscillator by Ogilvie et al. (1992), increasing excitation of I-AUG neurons decreases \( T_i \) and increases the slope of the inspiratory ramp (i.e., produces a “square wave” discharge), whereas increasing excitation of I-DEC neurons lengthens \( T_i \) (i.e., produces apneusis). It is possible that microinjection of DLH into the pre-BötzC stimulated this population of pre-I neurons, which in turn synthetically altered the activity of other inspiratory-modulated neurons (i.e., I-AUG and/or I-DEC) to produce the rapid series of high-amplitude, rapid rate of rise, short-duration inspiratory bursts or the tonic discharge of phrenic motor output seen in our experiments.

Involvement of excitatory amino acids in the generation and modulation of respiratory rhythm

Our findings are consistent with previous reports examining the involvement of excitatory amino acids in the generation and modulation of respiratory rhythm from in vitro neonatal rat brain stem–spinal cord and medullary slice preparations. In the in vitro brain stem–spinal cord preparation, Greer et al. (1991) have shown that bath application of NMDA and non-NMDA agonists increases respiratory burst frequency in cranial and spinal motoneurons in a dose-dependent manner. At the higher doses, the increase in respiratory burst frequency was replaced by a tonic discharge of cranial and spinal motoneuron pools, which obscured any evidence of rhythmic discharge. Conversely, bath application of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) reversibly decreased the frequency of respiratory bursts in a dose-dependent manner, abolishing rhythmic activity at the higher doses. Blockade of NMDA receptors was ineffective in altering respiratory burst frequency, suggesting that excitatory amino acid transmission mediated by non-NMDA receptor activation is necessary for respiratory rhythm generation in vitro. Furthermore, in the in vitro medullary slice preparation, unilateral microinjection of CNQX into the pre-BötzC reduced respiratory burst frequency and amplitude of hypoglossal motoneurons bilaterally in a dose-dependent manner, blocking respiratory oscillations at the higher doses (Funk et al. 1993). These findings demonstrate that non-NMDA receptor activation of neurons located in the pre-BötzC is essential for respiratory rhythm generation in vitro.

Much less is known about the role of this region in respiratory rhythm generation in vivo. Abrams et al. (1991) have shown that bilateral blockade of NMDA and non-NMDA re-
ceptors in the ventrolateral medulla (presumably in the rVRG/pre-BoëtC) of the cat produced a decrease in tidal volume and respiratory minute volume, which progress to apnea. More recently, Ramirez et al. (1998) have demonstrated that unilateral blockade of presynaptic N-type calcium channels or sodium channels in the pre-BoëtC of the cat produced a decrease in phrenic nerve activity or apnea and that bilateral blockade of sodium channels irreversibly abolished all respiratory rhythmic output. Additionally, Koshiya and Guyenet (1996) have shown that activation of GABA_A receptors in a region of the ventrolateral medulla that overlaps the rVRG and pre-BoëtC produced a generalized blockade of respiratory outflow (phrenic, vagal, and hypoglossal) in the rat.

In our experiments, we found that unilateral microinjection of DLH into the pre-BoëtC increases respiratory burst frequency (i.e., a rapid series of high-amplitude, short-duration bursts) or produces tonic discharge of phrenic motor output (i.e., a tonic excitation of phrenic neurogram output). We are not the first to describe the production of tonic excitatory effects on inspiratory activity in response to activation of excitatory amino acid receptors in the medulla. In addition to the findings of Greer et al. (1991) in the in vitro brain stem–spinal cord preparation, Bongianni et al. (1993) demonstrated that unilateral microinjection of DLH into the “apnoea region” of the rostral ventrolateral medulla (RVLM) (described by Budzinska et al. 1985) elicited both rhythmic and nonrhythmic excitatory effects on inspiratory activity. Although a portion of the apnoea region examined by Bongianni et al. overlaps our sites in the pre-BoëtC, they report only tonic excitatory effects, whereas we report modulations of both frequency and pattern (i.e., a rapid series of high-amplitude, short-duration bursts) in addition to the tonic excitatory response. One possible explanation for this discrepancy is that Bongianni et al. used a much higher concentration of DLH (160 mM) than that used in our study (10 mM); the volumes used in both studies are similar. As a result, it is possible that their microinjections of DLH produced a more intense depolarization in all sites examined, thus shifting putative conditional pacemaker neurons to a tonic state of action-potential generation.

In these experiments, we demonstrate that microinjection of DLH into a discrete region of the pre-BoëtC produced high-amplitude, rapid rate of rise, short-duration bursts. These characteristics are similar to the bursts produced by severe brain hypoxia and by pontomedullary transection, which have been considered to be gasps or gasplike activity (St. John and Knuth 1981). It should be noted that the high-amplitude, short-duration bursts produced by microinjection of DLH into the pre-BoëtC, in this study, were rapid with a short \( T_e \). Further, in our experiments, the pontomedullary junction was intact, and our cats were maintained hyperoxic (arterial \( P_{O2} \geq 200 \text{ mmHg} \)) throughout the experimental protocol. To our knowledge, these data are the first to show bursting activity with gasplike characteristics evoked by chemical stimulation of a discrete region of the medullary respiratory network. Previous work from St. John and colleagues (1984, 1985) has identified a region in the lateral tegmental field (LTF) of the medulla, which they propose is critical for the expression of gasping. They have shown that electrical stimulation in this region during gasping evokes premature gasps and that lesioning this region eliminates gasping, although neither maneuver alters the eupneic breathing pattern. In contrast, we demonstrate that direct chemical excitation of neurons in a discrete region located in the pre-BoëtC can modify both inspiratory timing and patterning to produce a gasplike inspiratory output. Clearly, our maneuver alters the eupneic breathing pattern, whereas those of St. John et al. (1984, 1985) do not. It is also clear that St. John et al. stimulate a region in the LTF that is anatomically distinct from the pre-BoëtC. In fact, the sites described by St. John et al. (1984) in the LTF are located dorsal, medial, and caudal to our sites in the pre-BoëtC. Thus these experiments do not settle the issue of whether there is a single gasping pattern generator and where it is located. They do indicate that highly discrete stimulation of what appears to be the principal respiratory rhythm generator may elicit “gasplike” outputs in the phrenic nerve.

**Effects of chemical stimulation in the pre-BoëtC on arterial blood pressure**

In most of our sites, the augmentation of inspiratory output was accompanied by marked pressor or depressor responses; however, we did encounter sites in which no blood pressure changes were observed. Bongianni et al. (1993) similarly reported marked pressor responses; however, in their experiments, depressor responses were fairly small. It is unlikely that the large increases in blood pressure reported in both our study and that of Bongianni et al. resulted from direct stimulation of sympathoexcitatory vasomotor neurons located in the RVLM. Anatomically, the region containing the C1 neuronal population (McAllen and Dampney 1989) is located rostral and ventral to the pre-BoëtC. On the basis of some preliminary experiments (Solomon et al. 1997), we propose that the increases in blood pressure observed in our study resulted from alterations in respiratory modulation of sympathetic activity. This proposal is supported by other recent neuroanatomic studies using intracellular biocytin injections demonstrating that inspiratory neurons originating in the rostral rVRG (some in the vicinity of the pre-BoëtC) project directly onto tyrosine hydroxylase-immunoreactive neurons located in the RVLM (Piilowsky et al. 1994), suggesting that these respiratory neurons may provide direct synaptic input to RVLM sympathoexcitatory neurons.

Our data provide no insight into the mechanism responsible for the production of the depressor responses reported here. Whether respiratory neurons originating in this region provide inhibitory synaptic inputs to sympathoexcitatory neurons of the RVLM or project directly to depressor regions of the caudal ventrolateral medulla remains to be determined.

**Conclusions**

In conclusion, we have demonstrated that increases in respiratory burst frequency and tonic discharge of inspiratory output, both of which have been seen in vitro, as well as modulation of burst pattern can be produced by local perturbations of excitatory amino acid neurotransmission in the pre-BoëtC in vivo. Thus this study provides evidence for a role of the pre-BoëtC in both generation and modulation of respiratory rhythm in vivo. Our findings, therefore, are consistent with the proposed role of this region as the locus for respiratory rhythm generation.

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