Influence of respiratory network drive on phrenic motor output evoked by activation of cat pre-Bötzinger complex

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Submitted 1 July 2002; accepted in final form 17 September 2002

Solomon, Irene C. Influence of respiratory network drive on phrenic motor output evoked by activation of cat pre-Bötzinger complex. Am J Physiol Regul Integr Comp Physiol 284: R455–R466, 2003; 10.1152/ajpregu.00395.2002. We have previously demonstrated that microinjection of DL-homocysteic acid (DLH), a glutamate analog, into the pre-Bötzinger complex (pre-BöC) can produce either phasic or tonic excitation of phrenic nerve discharge during hyperoxic normocapnia. Breathing, however, is influenced by input from both central and peripheral chemoreceptor activation. This influence of increased respiratory network drive on pre-BöC-induced modulation of phrenic motor output is unclear. Therefore, these experiments were designed to examine the effects of chemical stimulation of neurons (DLH; 10 mM; 10–20 nl) in the pre-BöC during hyperoxic modulation of CO₂ (i.e., hypcapnia and hypocapnia) and during normocapnic hypoxia in chloralose-anesthetized, vagotomized, mechanically ventilated cats. For these experiments, sites were selected in which unilateral microinjection of DLH into the pre-BöC during baseline conditions of hyperoxic normocapnia [arterial PO₂ (PaO₂)] = 37–43 mmHg; n = 22] produced a tonic (nonphasic) excitation of phrenic nerve discharge. During hypcapnia (PaCO₂ = 59.7 ± 2.8 mmHg; n = 17), similar microinjection produced excitation in which phasic respiratory bursts were superimposed on varying levels of tonic discharge. These DLH-induced phasic respiratory bursts had an increased frequency compared with the preinjection baseline frequency (P < 0.01). In contrast, during hypocapnia (PaCO₂ = 29.4 ± 1.5 mmHg; n = 11), microinjection of DLH produced nonphasic tonic excitation of phrenic nerve discharge that was less robust than the initial (normocapnic) response (i.e., decreased amplitude). During normocapnic hypoxia (PaCO₂ = 38.5 ± 3.7; arterial PO₂ = 38.4 ± 4.4; n = 8) microinjection of DLH produced phrenic excitation similar to that seen during hypcapnia (i.e., increased frequency of phasic respiratory bursts superimposed on tonic discharge). These findings demonstrate that phrenic motor activity evoked by chemical stimulation of the pre-BöC is influenced by and integrates with modulation of respiratory network drive mediated by input from central and peripheral chemoreceptors.

respiratory rhythm generation; neural control of breathing; hypcapnia; hypocapnia; hypoxia

THE PRE-BÖTZINGER COMPLEX (pre-BöC) is hypothesized to be the primary locus of respiratory rhythm generation in mammals (20, 24). Activation of this region in vivo has been demonstrated to increase the frequency of inspiratory bursts (5, 14, 28, 34) and produce tonic excitation of inspiratory motor activity (5, 28), while selective neuronal destruction of this region in vivo abolishes normal breathing (9) and the tachypnea response elicited by focal activation (34). Although anatomic specificity within the pre-BöC appears to explain some patterns of phasic modulation of inspiratory motor activity (i.e., rapid series of high-amplitude, rapid rate of rise, short-duration bursts) in vivo (16, 28), other mechanisms by which focal activation of the pre-BöC produces phasic vs. tonic excitation of respiratory motor output have not been examined.

One possible mechanism for different response types elicited by activation of this region might be the level of intrinsic excitability of pre-BöC rhythm-generating neurons. It has been proposed that the rhythm-generating neurons located in the pre-BöC receive synaptic inputs that synchronize and modify the basic rhythm generator by affecting intrinsic membrane conductances of presumptive rhythmogenic neurons (8, 19, 20, 24, 25), which in turn provide rhythmic drive to the respiratory network during the inspiratory phase of network activity (4, 8, 12, 20, 23–25). Thus the level of membrane depolarization of pre-BöC rhythm-generating neurons would vary with the level of overall respiratory network drive, which in vivo is influenced by input from both central and peripheral chemoreceptor activation.

In our previous experiments in the anesthetized cat, unilateral microinjection of the glutamate analog DL-homocysteic acid (DLH) into the pre-BöC produced excitation of phrenic motor output that exhibited either increased phasic burst frequency or tonic (i.e., nonphasic) discharge (28). Similar modulation of expiratory motor output in response to unilateral microinjection of DLH into the pre-BöC has also been observed in this animal model (26), confirming that activation of the pre-BöC is capable of eliciting both phasic and tonic (i.e., nonphasic) excitation of respiratory motor activity. Our previous studies, however,
were conducted under hyperoxic, normocapnic conditions; thus the influence of modified (i.e., increased or decreased) respiratory network drive on this DLH-induced pre-BötC-mediated excitation of respiratory motor output was not examined. Therefore, the purpose of this study was to specifically examine the effects of microinjection of DLH into the same site in the pre-BötC on phrenic motor output during modulation of CO₂ (i.e., hypercapnia and hypocapnia) and O₂ (i.e., hypoxia).

It has previously been demonstrated that respiratory network drive can influence the pattern of expression (i.e., phasic vs. tonic) of inspiratory and expiratory motoneuron discharges (22). In the absence of respiratory rhythm (produced by hypocapnia), for example, tonic excitation of respiratory motoneuron discharge is observed. As chemical drive (i.e., increasing CO₂ or hypoxia) is increased, however, phasic activity replaces tonic firing. Thus, if the level of intrinsic excitability of pre-BötC rhythm-generating neurons plays a role in determining the response type (i.e., phasic or tonic) elicited by DLH-induced activation of this region, then alterations in respiratory network drive should produce a predictable modulation of phrenic motor output elicited by chemical activation of this region. I hypothesized that repeated chemical activation of the same site in the pre-BötC will evoke different responses in phrenic motor output, which are dependent on the level of respiratory network drive. I further hypothesized that during increased respiratory network drive, activation of the pre-BötC would elicit an increase in the frequency of phasic phrenic bursts, while during reduced respiratory network drive, activation of the pre-BötC would elicit solely nonphasic tonic excitation of phrenic motor output.

**METHODS**

**General.** All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in compliance with the Animal Welfare Act and in accordance with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings” (1). A detailed description of the general methods has been published previously (28).

In brief, anesthesia was induced in adult cats (3.3–4.7 kg; n = 22) with halothane (5%) in oxygen and maintained with intravenous α-chloralose (initial 35–50 mg/kg; supplemental 3–5 mg/kg). The adequacy of anesthesia was regularly verified by absence of a withdrawal reflex (in the unparalyzed state) or blood pressure response (during muscular paralysis) to a noxious paw pinch. If the cat withdrew its limb during the absence of paralysis or if an increase in blood pressure was evoked, additional anesthesia was given. The right brachial vein and both brachial arteries were cannulated for administration of drugs, measurement of arterial blood pressure (Statham transducer, P23XL), and sampling of arterial blood. The trachea was cannulated, the cat was vagotomized bilaterally (to eliminate the influence of cardiopulmonary afferent input to the central respiratory network), and the lungs were mechanically ventilated with 40% O₂ in a balance of N₂. Bilateral pneumothorax were established, and the expiratory outlet of the ventilator was placed under 2–3 cmH₂O to prevent collapse of the lungs during expiration. The cat was then paralyzed with vecuronium bromide (0.2–0.4 mg/kg iv), supplemented as needed. The dorsal surface of the brain stem was exposed, and the C₂ rootlet of one or both phrenic nerves was isolated for recording. Raw phrenic nerve discharge was amplified (10,000 times) and band-pass filtered from 100 Hz to 10 kHz; the filtered signal was rectified, and a moving average was obtained using a third-order Paynter filter with a 100-ms time constant.

**Experimental protocol.** I examined the effects of DLH-induced activation of neurons located in the pre-BötC on phrenic nerve discharge during hyperoxic (PO₂ ≥ 175 mmHg) modification of CO₂ and during normocapnic hypoxia. Sites in the pre-BötC were initially localized using predetermined stereotaxic coordinates relative to the calamus scriptorius, functionally identified using DLH (28), and histologically confirmed after completion of the experiments. In all experiments, spontaneous phasic phrenic nerve activity was observed under hyperoxic, normocapnic conditions before functional identification. For these experiments, only functionally identified pre-BötC sites in which unilateral microinjection of DLH (10 mM; ≈20 nl; Sigma-Aldrich Chemical, St. Louis, MO) produced a tonic (nonphasic) excitation of phrenic nerve discharge under hyperoxic, normocapnic conditions were selected. To assess repeatability of this response, control experiments (n = 5) were conducted in which microinjection of DLH was repeated in the same site at least three times under hyperoxic, normocapnic conditions. In the remaining experiments, after functional identification of pre-BötC sites, one of the following changes in the ventilation parameters was made: 1) the fraction of O₂ in the inspired gas mixture was increased to 5 or 7% (hypercapnia), 2) the frequency of the ventilator was increased (hyperventilation/hypocapnia), or 3) the fraction of O₂ in the inspired gas mixture was decreased to 12–14% O₂ in a balance of N₂ (i.e., peripheral chemoreflex). The DLH microinjection was then repeated using the same volume used for functional identification. In all experiments, the volume of injectate was measured by observing the displacement of the fluid meniscus using a microscope equipped with an eyepiece reticle. Typically, the ventilation parameters were changed two or three times at each site before returning back to control levels to demonstrate recovery of the initial response. Changes in ventilation parameters were made in a randomized order, and at least 15 min of recovery were allowed before moving to the next trial. Under each condition, arterial PO₂ (Pao₂), Pco₂ (Paco₂), and pH were measured (Radiometer ABL-500) immediately preceding microinjection of DLH. To control for nonspecific effects, equivalent volumes of saline or larger volumes (≥120 nl) of 2% Fast green dye, which was used to mark injection sites, were microinjected into all sites. At the end of each experiment, the brain stem was removed for subsequent histological analysis to confirm that the injection site was in the pre-BötC (see Location of injection sites).

**Data acquisition and analysis.** Both raw and averaged phrenic nerve discharge were recorded on tape (Vetter, model 4000A) and on a chart recorder (Astro-Med, model MT95K2) throughout the experimental protocol. Appropriate segments of data were then transferred to a Macintosh PowerBook 3400c computer for offline analyses (PowerLab, Chart 3.6.1, ADInstruments).

Peak amplitude of integrated phrenic nerve discharge, inspiratory duration (T₁), expiratory duration (Tₑ), and frequency of phasic phrenic bursts were determined in response to unilateral microinjection of DLH into the pre-BötC under each of the ventilation conditions described above. Preinjec-
tion baseline values were determined by averaging the values obtained for the 60-s period preceding DLH microinjection. Response values were determined as the peak change from preinjection baseline values for a tonic nonphasic excitation of phrenic nerve discharge or by averaging the values obtained for five consecutive breathing cycles displaying the greatest change from preinjection baseline values for phasic phrenic nerve discharge responses. Further, for tonic nonphasic excitation of phrenic nerve discharge, \( T_t \) represents the duration of tonic firing, and \( T_h \) was not determined. Amplitude of integrated phrenic nerve discharge and frequency of phasic phrenic bursts are reported as a percent change from preinjection baseline levels of discharge, which were set at 100% in each cat. The onset latency for DLH-induced responses was measured from the beginning of microinjection.

All values are reported as means ± SE. Responses to DLH microinjection are presented as paired data. Student’s paired \( t \)-tests, the paired nonparametric Wilcoxon signed-rank test, or two-way repeated measures ANOVA, followed by Scheffé’s post hoc test, as appropriate, were used to determine statistical significance, for which the criterion level was set at \( P < 0.05 \).

RESULTS

General effects of DLH microinjection. Unilateral microinjection of DLH into 22 sites in the pre-Bo\( \ddot{t} \)C produced a nonphasic tonic excitation of phrenic nerve discharge under hyperoxic, normocapnic conditions (\( \text{PaO}_2 = 189.6 \pm 5.6 ~\text{mmHg}; \text{PaCO}_2 = 39.4 \pm 2.3 ~\text{mmHg} \)). This response was characterized by an abrupt rise in phrenic nerve discharge to a plateau level, which in some cases gradually decayed, and had durations ranging from 20 to 165 s. In general, the peak amplitude of integrated phrenic nerve discharge at the onset of the response was higher or the same as that seen during preinjection baseline phrenic bursts, and recovery consisted of either a gradual return of phasic phrenic bursts or a transient postexcitatory depression of phrenic nerve discharge. In some cases (\( n = 7 \)), phasic phrenic bursts returned as tonic activity began to wane (i.e., before complete cessation of tonic activity).

Repeatability of the DLH-induced response. Repeated microinjection of DLH into the same site in the pre-Bo\( \ddot{t} \)C without modulation of the ventilation parameters (i.e., maintained under hyperoxic, normocapnic conditions; \( n = 5 \)) demonstrated that the DLH-induced response was reproducible within the same site (Fig. 1). In general, at least 15 min was allowed for recovery before attempting to demonstrate repeatability, and at least three microinjections were made into the same site. An example of the results obtained from one of these experiments is provided in Fig. 1A, and summary data describing the changes in peak amplitude of integrated phrenic nerve discharge and \( T_t \) for each microinjection trial from all five experiments are illustrated in Fig. 1B. As shown, repeated microinjection of DLH into the same site in the pre-Bo\( \ddot{t} \)C elicited a reproducible increase in phrenic nerve discharge (Fig. 1A), with no differences observed in either the DLH-induced increase in peak amplitude of integrated phrenic nerve discharge or the DLH-induced increase in \( T_t \) (Fig. 1B).

Effects of modulation of \( \text{CO}_2 \) on the DLH-induced response. I examined the effects of altering inspired \( \text{CO}_2 \) on phrenic nerve activity in response to DLH-induced activation of the pre-Bo\( \ddot{t} \)C. Responses from 17 sites were examined during hypercapnia, and responses from 11 sites were examined during hypocapnia. Arterial blood gases and pH, which were measured immediately preceding unilateral microinjection of DLH into the pre-Bo\( \ddot{t} \)C during normocapnia (baseline), hypercapnia, and hypocapnia, are provided in Table 1, and an example of the results obtained from one experiment examining the effects of both hypercapnia.
Table 1. Arterial blood gases and pH during systemic modulation of CO₂

<table>
<thead>
<tr>
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<th>PaO₂</th>
<th>PaCO₂</th>
<th>pHa</th>
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<tbody>
<tr>
<td>Normocapnia</td>
<td>187.3 ± 5.7</td>
<td>38.3 ± 2.2</td>
<td>7.37 ± 0.01</td>
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<tr>
<td>Hypercapnia</td>
<td>186.9 ± 5.3</td>
<td>59.7 ± 2.8*</td>
<td>7.24 ± 0.01*</td>
</tr>
<tr>
<td>Hypocapnia</td>
<td>184.8 ± 6.1</td>
<td>29.4 ± 1.5*</td>
<td>7.41 ± 0.01*</td>
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</table>

Values are means ± SE. PaO₂, arterial P O₂; PaCO₂, arterial P CO₂; pHa, arterial pH. *Statistically different (P < 0.05) from control (hyperoxic, normocapnia).

In these experiments, during normocapnia, unilateral microinjection of DLH into the pre-BöC produced a nonphasic tonic excitation of phrenic nerve discharge in each of the sites examined (as described above). Hypercapnia elicited an increase in the amplitude of phrenic bursts with little or no effect on burst frequency (i.e., preinjection baseline). During hypercapnia, similar microinjection of DLH into the same sites in the pre-BöC produced excitation of phrenic nerve discharge in which phasic bursts were superimposed on varying levels of tonic activity. The onset latency for DLH-induced excitation of phrenic nerve discharge was similar during normocapnia and hypercapnia (P > 0.05), with responses being observed within 1–3 s from the beginning of microinjection.

Data were obtained during either moderate or severe hypercapnia, which was produced by adding 5 or 7% CO₂ to the inspired gas, respectively. Examples demonstrating the effects of moderate and/or severe hypercapnia on the DLH-induced response are provided in Figs. 2 and 3. Regardless of the severity of the hypercapnic challenge, microinjection of DLH into the pre-BöC during hypercapnia modified the DLH-induced response, such that it included phasic phrenic bursts. In contrast, the level of DLH-induced tonic activity appeared to be dependent on the severity of the hypercapnic challenge, such that higher levels of CO₂ were accompanied by lower levels of underlying tonic discharge. Examples demonstrating the effect of the severity of hypercapnia on the magnitude of the DLH-induced underlying tonic activity during both moderate and severe hypercapnia can be seen in Fig. 3 (also see Fig. 2B for effects of moderate hypercapnia).

In addition, the duration of the DLH-induced tonic excitation also appeared to be dependent on the severity of the hypercapnic challenge, such that shorter durations of underlying tonic discharge were observed at higher levels of CO₂ (Fig. 4A). Summary data illustrating these effects of moderate (n = 10) and severe (n = 7) hypercapnia on the DLH-induced changes in peak amplitude and T₁ of the tonic component of integrated phrenic nerve discharge are provided in Fig. 4A. For these experiments, comparisons were made between the DLH-induced responses during normocapnia and hypercapnia as well as between the two different levels of hypercapnia.

Although microinjection of DLH into the pre-BöC during hypercapnia elicited phasic phrenic bursts, both the amplitude and the timing of these phasic bursts were different from those recorded during the preinjection baseline. This DLH-induced modulation appeared to be independent of the severity of the hypercapnic challenge, and therefore these data have been combined for statistical analyses. Summary data and hypocapnia on DLH-induced activation of the pre-BöC is provided in Fig. 2.
illustrating the DLH-induced changes in peak amplitude of integrated phasic phrenic nerve discharge, frequency of phasic bursts, $T_I$, and $T_E$ for all 17 of the hypercapnia experiments are provided in Fig. 5. In general, microinjection of DLH into the pre-Bo$\ddot{O}$tC elicited a small increase in the peak amplitude of integrated phasic phrenic nerve activity (Fig. 5A; $P < 0.05$), although a decrease in the peak amplitude of integrated phasic phrenic nerve activity was observed in 5 of the 17 sites examined (including the responses shown in Figs. 2B and 3A2). In addition, the DLH-induced phasic bursts had an increased frequency compared with the preinjection baseline frequency (Fig. 5B; $P < 0.01$). This increased frequency resulted predominantly from a reduction in $T_E$ (Fig. 5D; $P < 0.001$) although a decrease in $T_I$ (Fig. 5C; $P < 0.05$) was also observed in 14 of the 17 experiments.

I also examined the effects of hypocapnia on the DLH-induced response in 11 sites in the pre-Bo$\ddot{O}$tC. For these experiments, inspired CO$_2$ was reduced by increasing the rate of the ventilator. In two experiments, the level of CO$_2$ was reduced to produce depression of phrenic nerve activity of 40–50% and then the level of CO$_2$ was further reduced to produce phrenic apnea (i.e., apnic threshold). An example of the results obtained from one of these experiments is provided in Fig. 2. In the remaining experiments, the level of CO$_2$ was reduced to produce phrenic apnea. During phrenic apnea, spurious action potential discharges were observed in some cases.

In these experiments, during normocapnia, unilateral microinjection of DLH into the pre-Bo$\ddot{O}$tC produced a nonphasic tonic excitation of phrenic nerve discharge in each of the sites examined (as described above). During hypocapnia, similar microinjection of DLH into the same sites in the pre-Bo$\ddot{O}$tC also produced a nonpha-
increased the amplitude of integrated phrenic nerve discharge \((A)\). In seven of these experiments, there was an incapable was not statistically signi
terning characteristics of phrenic nerve discharge. During hypercap-
discharge compared with the DLH-induced response
increase in peak amplitude of integrated phrenic nerve discharge during normocapnia \((P\) \(\text{Hypoxia} 8 189.6 48.4 38.4\)

Effects of modulation of \(O_2\) on the DLH-induced response. I examined the effects of altering inspired \(O_2\) on phrenic nerve activity in response to DLH-induced activation of the pre-\(\text{BöC}\). Responses from eight sites were examined during systemic hypoxia (i.e., peripheral chemoreflex). Arterial blood gases and pH immediately preceding unilateral microinjection of DLH into the pre-\(\text{BöC}\) during hypoxia (baseline) and hypocapnia are provided in Table 2, and an example of the results obtained from one of these experiments is provided in Fig. 6.

In these experiments, during hyperoxic normocapnia, unilateral microinjection of DLH into the pre-\(\text{BöC}\) produced a nonphasic tonic excitation of phrenic nerve discharge in each of the sites examined (as described above). Systemic hypoxia (12–14% \(O_2\)) elicited an increase in the amplitude of phrenic bursts with little or no effect on burst frequency (i.e., preinjection baseline). During systemic hypoxia, similar microinjection of DLH into the same sites in the pre-\(\text{BöC}\) produced excitation of phrenic nerve discharge in which phasic bursts were superimposed on varying levels of tonic activity. The onset latency for DLH-induced excitation of phrenic nerve discharge was similar during hyperoxic normocapnia and systemic hypoxia \((P > 0.05)\), with responses being observed within 1–3 s from the beginning of microinjection. In all experiments, microinjection of DLH into the pre-\(\text{BöC}\) during systemic hypoxia modified the DLH-induced response, such that it included phasic phrenic bursts. Although microinjection of DLH into the pre-\(\text{BöC}\) during systemic hypoxia elicited phasic phrenic bursts, the patterning of these phasic phrenic bursts was quite variable and typically included a high-amplitude, short-duration burst component, such as those associated with the augmented burst (i.e., sigh) pattern. Examples of augmented bursts produced in response to microinjection of DLH into the pre-\(\text{BöC}\) during hypoxia are presented in Fig. 6, with Fig. 6B providing an expanded time scale. It

Table 2. Arterial blood gases and pH during systemic modulation of \(O_2\)

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<tr>
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<th>(n)</th>
<th>(P_{\text{aO}_2}), mmHg</th>
<th>(P_{\text{aCO}_2}), mmHg</th>
<th>(pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperoxia</td>
<td>8</td>
<td>189.6 ± 5.8</td>
<td>39.3 ± 2.2</td>
<td>7.38 ± 0.01</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>8</td>
<td>38.4 ± 4.4*</td>
<td>38.5 ± 3.7</td>
<td>7.37 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Statistically different \((P < 0.05)\) from control (hyperoxic, normocapnia).
should be noted that the augmented bursts shown in Fig. 6B1 are a subset of bursts from the response illustrated in Fig. 6A2, while the augmented bursts shown in Fig. 6B2 were obtained from an experiment in a different animal (full response not shown).

In addition to the patterning changes noted, the timing of these bursts was also different from those recorded during the preinjection baseline. Summary data illustrating the DLH-induced changes in peak amplitude of integrated phrenic nerve discharge, frequency of phasic respiratory bursts, T1, and T2 for all eight of the systemic hypoxia experiments are illustrated in Fig. 7. Overall, microinjection of DLH into the pre-BötC during systemic hypoxia was ineffective in changing the peak amplitude of integrated phasic respiratory bursts from the response shown in Fig. 6A1, while an increase in the peak amplitude of integrated phasic respiratory bursts was observed in five of the eight sites examined (including the response shown in Fig. 6A4), while a decrease in the peak amplitude of integrated phasic respiratory bursts was observed in the remaining three sites examined. It should also be noted that the high-amplitude, short-duration burst components are not included in the measurement of the peak amplitude of integrated respiratory bursts. The DLH-induced respiratory bursts had an increased frequency compared with the preinjection baseline frequency (Fig. 7B; P < 0.01). This increased frequency resulted from a reduction in both T2 (Fig. 7D; P < 0.01) and T1 (Fig. 7C; P < 0.01).

Location of injection sites. The distribution of sites in which DLH was microinjected into the pre-BötC is shown in Fig. 8. As landmarks for identifying the rostrocaudal level of the pre-BötC, I 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. All sites in the pre-BötC were identified with reference to the caudal pole of the retrofacial nucleus.
Histological analyses revealed that all microinjection sites functionally identified as pre-BötC were located within the anatomic boundaries described for the pre-BötC in adult cat (6, 18, 21, 28). The microinjection sites were located within 320 μm (i.e., 0–320 μm caudal) of the caudal pole of the retrofacial nucleus in the rostrocaudal plane, 3.72–4.04 mm lateral to midline in the mediolateral plane, and 4.20–4.36 mm ventral to the dorsal surface of the medulla in the dorsoventral plane. No differences were detected in the location of sites used for experiments examining repeatability of the DLH-induced response (Fig. 8A) vs. those examining the effects of modulation of CO_2 and hypoxia on the DLH-induced response (Fig. 8B).

**DISCUSSION**

In the present study, I have demonstrated that modulation of respiratory network drive alters the phrenic motor output response elicited by DLH-induced activation of the pre-BötC in vivo. I have shown that although chemical stimulation of the pre-BötC under hyperoxic normocapnic conditions can produce a tonic (nonphasic) excitation of phrenic nerve discharge, during increased respiratory network drive, produced by either hypercapnia or hypoxia, chemical stimulation of this region elicits phasic phrenic bursts that are superimposed on varying levels of tonic phrenic nerve discharge. Furthermore, these phasic bursts exhibit an increased burst frequency compared with the preinjection baseline burst frequency. Conversely, during decreased respiratory network drive produced by hypcapnia, chemical stimulation of the pre-BötC evokes only a low-amplitude nonphasic tonic increase in phrenic nerve discharge. To my knowledge, this is the first demonstration that pre-BötC-induced excitation of phrenic motor output can be modulated by alterations in respiratory network drive. I interpret these findings to suggest that phrenic motor activity evoked by chemical stimulation of the pre-BötC is influenced by and integrates with modulation of respiratory network drive mediated by input from central and peripheral chemoreceptors.

**Limitations of the current study.** There are four main limitations in the current investigation. First, the effects of altered respiratory network drive on the phrenic nerve discharge responses evoked by DLH-induced activation of the pre-BötC were examined in chloralose-anesthetized adult cats. Therefore, the patterns of evoked phrenic nerve activity observed in the current investigation (as well as in our previous study; 28) may have been influenced by the effects of anesthesia. It should be noted that anesthesia has previously demonstrated to depress respiratory network activity due to enhanced inhibitory synaptic interactions; thus it remains to be determined whether alterations in respiratory network drive would similarly modify DLH-induced pre-BötC-mediated phrenic nerve discharge responses in the unanesthetized (awake and/or decerebrate) state.

Second, because respiratory network drive was altered by systemic modulation of CO_2 and O_2 in the current experiments, neuronal excitability within the pre-BötC may have been modified by either direct or indirect mechanisms. Systemic hypercapnia and systemic hypoxia are well known to activate central and peripheral chemoreceptors, resulting in increased excitatory synaptic input to the brainstem respiratory centers. In addition, in vitro transverse medullary slices obtained from neonatal mice (postnatal days 0–22), an increase in the amplitude of excitatory synaptic potentials has been demonstrated in ~50% of pre-BötC inspiratory neurons in response to hypoxia (17). Although increased excitatory synaptic input mediated by enhanced release of excitatory neurotransmitters and neuromodulators in the pre-BötC can explain modulation of neuronal excitability within the pre-BötC during systemic hypercapnia and systemic hypoxia, recent studies have demonstrated intrinsic CO_2/H^+ and hypoxic chemosensitivity within the pre-BötC (11, 29, 31, 33), and presumptive rhythmogenic
pacemaker neurons appear to be the CO₂/H⁺- and hypoxia-chemosensitive neurons in this region (11, 33). The direct effects of focal modulation of CO₂/H⁺ and hypoxia in the pre-BoTC on DLH-induced responses, however, were not assessed in the current experiments; therefore, the contribution of intrinsic pre-BoTC chemosensitivity to modulation of the DLH-induced responses observed is unknown. It should be noted that during severe hypoxia that is sufficient to produce gasping, microinjection of DLH into functionally identified (during hyperoxic normocapnia) nonphasic tonic sites in the pre-BoTC produces frequency modulation of gasplike phrenic bursts (27). The level of hypoxia used in the current experiments, however, was not severe (which appears to be a prerequisite for intrinsic hypoxic chemosensitivity in the in vivo pre-BoTC; 29), and therefore, it is unlikely that intrinsic hypoxic chemosensitivity played a significant role in modulation of the DLH-induced responses observed (i.e., increased excitatory synaptic inputs would be primarily responsible). From the current experiments, however, I cannot exclude the possibility that intrinsic CO₂/H⁺ and/or hypoxic chemosensitivity within the pre-BoTC played a role, at least in part, in the modulation of the DLH-induced responses observed.

Third, although the pre-BoTC is a bilateral structure, activation of this region was restricted to a unilateral microinjection; thus the effects of bilateral activation of the pre-BoTC on phrenic nerve discharge (during hyperoxic normocapnia and altered respiratory network drive) were not examined. I believe, however, that the modulation of the DLH-induced responses observed in these experiments was specific to chemical stimulation of the pre-BoTC and did not result from spread of DLH to the adjacent BoTc complex (BoTC) or rostral ventral respiratory group (rVRG). Although I cannot exclude the possibility of spread, I suggest that this is unlikely. In our previous experiments, we have demonstrated that nonphasic tonic excitation of phrenic nerve discharge and modulation of phrenic burst frequency were only observed in response to chemical stimulation of the pre-BoTC (28), while similar activation of the adjacent BoTC and rVRG regions has been shown to elicit changes in only amplitude of phrenic nerve discharge (3, 5, 13, 14, 28). Furthermore, preliminary data suggest that increased respiratory network drive, produced by hypercapnia, attenuates the changes in amplitude of phrenic nerve discharge elicited by DLH-induced activation of these adjacent regions (unpublished observations). I cannot exclude, however, the possibility that microinjection of DLH had an effect on dendrites whose cell bodies were distant from the site of injection.

Finally, for the current experiments, I only selected sites in the pre-BoTC in which microinjection of DLH produced a nonphasic tonic excitation of phrenic nerve discharge under hyperoxic, normocapnic conditions. Although I often encountered sites in which DLH-induced activation of the pre-BoTC evoked other patterns of excitation of phrenic nerve discharge (as previously reported; 28), these sites were avoided because the DLH-induced response already included modulation of phrenic burst frequency. The sites not included in the current investigation were predominantly those in which DLH-induced excitation of phrenic nerve activity included a high-amplitude, short-duration burst component. When this type of response was encountered, the microinjection pipette was moved 200–300 µm rostral, as this response type is generally obtained from sites caudal to those in which tonic discharge is evoked (16). Thus, whether respiratory network drive influences these other pre-BoTC-mediated DLH-induced patterns of excitation of phrenic nerve discharge remains to be determined.

**Chemical activation of the pre-BoTC in vivo.** Previous in vivo studies, including work from our laboratory, have demonstrated an increase in the frequency of inspiratory bursts (5, 14, 26, 28, 34) as well as tonic (nonphasic) excitation of inspiratory motor activity (26, 28) in response to chemical stimulation of this region; however, these studies did not assess the effects of pre-BoTC activation during alterations in respiratory network drive. In our previous experiments in the anesthetized cat, we reported that microinjection of DLH into the pre-BoTC under hyperoxic normocapnic conditions elicited multiple patterns of excitation of phrenic nerve discharge, including tonic excitation with phasic respiratory bursts superimposed (28). Although in our previous experiments the cats were maintained normocapnic during the experimental protocol, the apneic threshold was not identified. Thus one possible explanation for our previous finding is that in some of our experiments, the cats may have been farther away from their apneic threshold, and therefore, overall respiratory network drive may have been relatively higher in those animals. It should be noted, however, that in sites in which DLH-induced activation of the pre-BoTC produces tonic excitation with phasic respiratory bursts superimposed during hyperoxic normocapnia, reducing CO₂ in some of these animals shifts the DLH-induced response to a nonphasic tonic excitation (unpublished observations), consistent with the effects of reducing CO₂ in the present investigation.

Although multiple patterns of excitation of phrenic nerve discharge have been elicited by chemical stimulation of the pre-BoTC in vivo (5, 14, 28), the precise mechanism(s) by which focal pre-BoTC activation produces phasic vs. tonic excitation of phrenic nerve discharge remain to be resolved. In our previous experiments, histological analyses revealed some degree of site specificity for some patterns of DLH-induced phasic activity (i.e., rapid series of high-amplitude, rapid rate of rise, short-duration bursts), but our histological analyses could not distinguish sites in which DLH-induced activation produced nonphasic tonic discharge from those that produced tonic discharge with phasic bursts superimposed (28). In the current experiments, DLH-induced activation of a single site in the pre-BoTC during increased respiratory network drive (i.e., hypercapnia and hypoxia) elicited modulation of phasic phrenic burst frequency, which was not observed in...
response to similar activation during either baseline conditions (i.e., hyperoxic normocapnia) or hypocapnia; thus modulation of respiratory network drive appears to be one mechanism capable of influencing the response type evoked by repeated activation of a single site in the pre-BötC. The current experiments, however, did not investigate the precise cellular mechanism(s) within the pre-BötC responsible for this modulation of the DLH-induced response.

**DLH-induced tonic (nonphasic) phrenic nerve discharge.** In our previous experiments, we suggested that nonphasic tonic excitation of phrenic nerve discharge in response to activation of the pre-BötC may result from a shift in the membrane potential of presumptive rhythmonic pre-BötC neurons from a level of quiescent or phasic (or oscillatory bursting) activity to a more depolarized level, leading to tonic (or beating) action potential generation, a response similar to that observed by depolarizing the voltage-dependent pacemaker cells identified in the neonatal rodent pre-BötC (8, 12, 20, 24, 25). The pre-BötC in adult cat and rat, however, is characterized by a mixture of neurons exhibiting inspiratory-modulated, expiratory-modulated, and phase-spanning (including preinspiratory) discharge patterns (6, 10, 21, 32) and appears to contain neuronal elements that may be essential for respiratory rhythm generation (9, 18). Therefore, other possible explanations for the production of nonphasic tonic excitation of phrenic nerve discharge in response to activation of the pre-BötC must be considered. I suggest that nonphasic tonic excitation may, alternatively, result from DLH-induced stimulation of multiple classes of respiratory-modulated neurons located in this region, which in turn could lead to a reduction or loss of the synchronized phasic neuronal activity required for the generation or expression of phasic phrenic bursts. This explanation does not require that the phasic (or rhythmic) activity of the presumptive rhythmonic neurons be abolished but does suggest that the phasic (or rhythmic) activity of presumptive rhythmonic pre-BötC neurons may not be sufficient (or adequately synchronized) to allow for expression of phasic phrenic motor activity. This may be similar to the ectopic bursts observed between phasic hypoglossal nerve discharges in most of the presumptive rhythmonic pacemaker neurons tested in the pre-BötC of neonatal rats (postnatal days 0–3) in vitro (7). Thus, if this prediction is correct, it could explain why during decreased respiratory network drive (i.e., hypocapnia), microinjection of DLH into the pre-BötC evoked nonphasic tonic phrenic nerve activity instead of phasic discharge, which might be expected (based on the hybrid “pacemaker-network” model of respiratory rhythm generation; 4, 7, 8, 12, 20, 23, 25). It is possible, however, that nonphasic tonic excitation, which was observed during both normocapnia and hypocapnia, may result from a combination of the above effects. From the current experiments, it is not clear which, if any, of these potential mechanisms is responsible for the production of nonphasic tonic excitation of phrenic nerve discharge in response to DLH-induced activation of the pre-BötC.

It should also be noted that the above explanations for DLH-induced generation of nonphasic tonic phrenic nerve discharge assume that the tonic discharge is not masking or occluding an underlying phasic phrenic nerve discharge. I do not believe that the DLH-induced “nonphasic” tonic excitation of phrenic nerve activity occludes phasic motoneuron output because in our previous experiments 1) DLH-induced nonphasic tonic excitation of phrenic nerve discharge was associated with both increased and decreased peak amplitude of tonic phrenic nerve discharge compared with peak amplitude of preinjection baseline phasic phrenic nerve discharge (26, 28, 29); 2) DLH-induced nonphasic tonic excitation was observed simultaneously in both inspiratory and expiratory motor outputs (26); and 3) DLH-induced nonphasic tonic excitation suppressed both the phasic (inspiratory modulated) and tonic components of sympathetic nerve discharge recorded from the preganglionic cervical sympathetic nerve (30).

**Modulation of DLH-induced phrenic nerve discharge by increased respiratory network drive.** During increased respiratory network drive produced by hypercapnia or hypoxia, DLH-induced activation of the pre-BötC would presumably affect the same populations of neurons described above. Under these conditions, however, stimulation of the pre-BötC could elicit a different effect on phrenic nerve discharge because changes in the strength of synaptic interactions and/or changes in activity of a population of respiratory neurons can modify respiratory rhythm and pattern (2, 15). In the current experiments, microinjection of DLH into the pre-BötC during increased respiratory network drive elicited predominantly frequency modulation of phasic phrenic nerve discharge. At first glance, it appears that these data are inconsistent with the hybrid pacemaker-network model of respiratory rhythm generation (4, 7, 8, 12, 20, 23, 25). Based on this model, it might be expected that during increased respiratory network drive, microinjection of DLH into the pre-BötC would evoke tonic, not phasic, phrenic nerve discharge (see explanation above). I do not believe that these data are inconsistent with the proposed hybrid pacemaker-network model of respiratory rhythm generation but may reflect a wide dynamic range of neuronal excitability of presumptive rhythmonic pre-BötC neurons (7) available for modulation of phrenic burst frequency. It should be noted, however, that in the adult anesthetized cat, “pacemaker” cells have not been identified nor has the behavior of individual presumptive rhythmonic pre-BötC neurons been examined in response to increased excitability within the pre-BötC.

With respect to the current findings, I suggest that during increased respiratory network drive, the presumptive rhythmonic pre-BötC neurons are closer to threshold for eliciting a rhythmonic (i.e., frequency modulation) response to DLH-induced activation of the pre-BötC. Further, under these conditions, DLH-induced activation of this region elicits less of a contribution of the respiratory-modulated, nonrhythmic
pre-BötzC neurons because these neurons are in a more excited state due to increased synaptic drive (a similar mechanism of reduced contribution to neuronal excitation might also explain the effects observed in response to DLH-induced activation on adjacent regions during hypercapnia; see above). If this prediction is correct, I would expect to see both frequency modulation of phasic phrenic bursts and a reduction in the level of tonic phrenic nerve discharge in response to microinjection of DLH into the pre-BötzC during increased respiratory network drive. In the current experiments, I found that during increased respiratory network drive, microinjection of DLH into the pre-BötzC consistently added a phasic component, which included frequency modulation, to varying levels of tonic phrenic nerve discharge. Furthermore, with higher levels of respiratory network drive, such as that produced by severe hypercapnia, the level of DLH-induced underlying tonic phrenic nerve discharge was substantially reduced.

In conclusion, I interpret the current findings to suggest that the basic rhythm-generating circuitry located in the pre-BötzC (20, 23, 24) is still responsive to activation during increased respiratory network drive and that the predominant response under these conditions is modulation of frequency of phrenic bursts. Furthermore, I suggest that the response elicited by activation of the pre-BötzC results not only from the level of intrinsic excitability of presumptive rhythmic pre-BötzC neurons but also the level of rhythmic synaptic drive to other classes of inspiratory-modulated neurons (including nonrhythmic pre-BötzC neurons) and the interaction of presumptive rhythmic pre-BötzC neurons with these other respiratory-modulated neurons.

In summary, the findings of the current investigation demonstrate that the phrenic motor output responses evoked by chemical stimulation of the pre-BötzC in vivo are strongly modulated by the excitatory state of the respiratory network. I suggest that increased respiratory network drive increases neuronal excitability of presumptive rhythmic pre-BötzC neurons and enhances rhythmic synaptic drive to other classes of inspiratory-modulated neurons (including nonrhythmic pre-BötzC neurons), resulting in modulation of the pre-BötzC-mediated DLH-induced response. The findings further indicate that the pre-BötzC has the potential to play a role in frequency modulation of phrenic motor output during increased respiratory network drive because chemical stimulation of this region under these conditions elicits an increase in frequency of phrenic bursts. Thus this study provides additional in vivo evidence for a role of this region in modulation of phasic respiratory activity.

The author thanks T. J. Halat for excellent technical assistance. This work was supported by National Heart, Lung, and Blood Institute Grant HL-60175.

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