Mylohyoid discharge of the in situ rat: a probe of pontile respiratory activities in eupnea and gasping

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Mylohyoid discharge of the in situ rat: a probe of pontile respiratory activities in eupnea and gasping. J Appl Physiol 108: 614–620, 2010. First published December 24, 2009; doi:10.1152/japplphysiol.00988.2009.—Our purpose was to characterize respiratory-modulated activity of the mylohyoid nerve. Since its motoneurons are in the trigeminal motor nucleus, mylohyoid discharge could serve as a probe of the role of pontile mechanisms in the generation of respiratory rhythms. Studies were performed in the decerebrate, perfused in situ preparation of the rat. Phrenic discharge was recorded as the index of the respiratory rhythm. In eupnea, the mylohyoid nerve discharged primarily during the inspiratory phase of the respiratory cycle. These results provide evidence that the mylohyoid nerve could serve as a probe of respiratory-modulated activity in the pons. The respiratory-modulated activities of the mylohyoid nerve discharge in gasping supports the concept that a release of pontile influences, underlies the neurogenesis of gasping. Results also provide additional support for our conclusion that activity of any single cranial nerve does not provide an accurate index of the type of respiratory rhythm, be it eupnea or gasping.

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NORMAL BREATHING involves the coordinated activation of cranial and spinal nerves (3). Inspiration is defined by the discharge of the phrenic nerve. However, for a normal filling of the lungs to occur in inspiration, activities of multiple cranial nerves are required to dilate and/or stiffen the oro- and nasopharynx and the larynx (2, 3). Expiratory airflow occurs passively during unstimulated breathing, when the lungs return to the functional residual capacity. Active expiration, which can be induced by increases in respiratory drive, involves activation of spinal nerves and contraction of intercostal and abdominal muscles. Cranial nerves may be active during both passive and active expiration to stabilize the larynx and upper airways (2, 3).

The coordinated and sequential activation of cranial and spinal nerves throughout the respiratory cycle is dependent on a complex circuit of neuronal activities in pons and medulla (3, 27). The role of pontile mechanisms in this activation has recently been reinforced by the finding that the timing of onsets of cranial compared with spinal nerves is lost following removal of pontile influences (24).

In addition to a role in determining the timing of the respiratory cycle, pontile mechanisms are hypothesized to play a role in the generation of the respiratory rhythm per se. This role might be expressed as part of a pontomedullary neural circuit and/or by neuronal mechanisms intrinsic to pons (24, 27, 34). Examination of such a role for pons in respiratory rhythm generation is timely as accumulating evidence establishes that no region of medulla, including the pre-Bötzinger complex, constitutes a unique region for generating normal breathing (see Refs. 26, 27 for review).

While motoneurons of most cranial nerves having a respiratory-modulated discharge pattern are located in medulla or at the pontomedullary junction, the mylohyoid nerve has its motoneurons in the trigeminal motor nucleus of pons (6, 28). The respiratory-modulated activities of the mylohyoid nerve have been little characterized, and no characterization in rodents has been performed. In the present study, we have undertaken a characterization of mylohyoid activity, including its responses to hypercapnia, hypoxia, and ischemia. Results provide a basis by which, in future experiments involving isolation of pons from medulla, mylohyoid discharge may serve as a probe of respiratory-rhythm generation by intrinsic pontile mechanisms.

METHODS

Experimental Preparations

Fifty-seven perfused preparations of the decerebrate juvenile rat were used. All procedures used in these studies have been approved by the Institutional Animal Care and Use Committee of Dartmouth College and Dartmouth Medical School. The Animal Resource Center at Dartmouth Medical School is an American Association for Accreditation of Laboratory Animal Care-approved facility.

Rats were of age 21–30 days and weight of 90–120 g. Under deep anesthesia with enflurane, rats were bisected caudal to the diaphragm and decerebrated at a precollicular level, as described previously (21, 30). Anesthesia was discontinued following decerebration. Preparations were maintained in hypothermia by immersion in ice-cold mock cerebrospinal fluid.

In four of the rats, influences from the peripheral chemoreceptors were removed by sectioning the vagi and the carotid sinus nerves. For the latter, all tissue was removed from the common and external carotid arteries and the internal carotid arteries were ligated and sectioned.

Isolation of Phrenic and Mylohyoid Nerves

The phrenic nerve was sectioned at the level of the diaphragm and dissected rostrally. From a ventral approach, the branches of the mylohyoid nerve were identified as these emerged from beneath the hyoid bone. With the use of a rongeur, the mylohyoid nerve was separated from the overlying tissues. The mylohyoid nerve was then freed from the underlying muscles. With the use of a rongeur, the caudal portion of the mandible was removed so that a length of the mylohyoid nerve was visible. The nerve was freed from the underlying muscle and sectioned peripherally (Fig. 1).
Perfusion of the Preparation

The descending aorta was cannulated and perfusion was commenced. The perfusate contained the following in distilled water: magnesium sulfate (MgSO₄; 1.25 mM), potassium phosphate (KH₂PO₄; 1.25 mM), potassium chloride (KCl; 3.0 mM), sodium bicarbonate (NaHCO₃; 2.4 mM), sodium chloride (NaCl; 125 mM), calcium chloride (CaCl₂; 2.5 mM), dextrose (10 mM), Ficoll 70 (0.1785 mM). Gallamine triethiodide was added to the perfusate to block neuromuscular transmission. The temperature of the perfusate as it entered the aorta was 31°C. Under control conditions, the perfusate was equilibrated with a gas mixture of 95% O₂-5% CO₂ (hyperoxic normocapnia).

Recording of Neural Activities

Efferent activities of the phrenic and mylohyoid nerves were recorded with suction electrodes. These activities were amplified, filtered (0.6–6.0 kHz), and integrated (50 ms time constant).

Changes in Respiratory “Drive” and “Pattern”

With the perfusate equilibrated with a hyperoxic-normocapnic gas mixture (95% O₂-5% CO₂), integrated phrenic discharge had an incrementing pattern, which is akin to eupnea (30).

Hypocapnia and hypercapnia were produced by changing the concentration of carbon dioxide that was added to the hyperoxic gas. Levels of oxygen were altered by mixing nitrogen and oxygen while keeping constant the percentage of carbon dioxide. Note that the perfusate, having no hemoglobin, has a much lower capacity for carrying oxygen than blood. Hence, while brain stem tissue is hypoxic when the preparation is perfused with a solution equilibrated with 95% O₂-5% CO₂ (35), this brain stem tissue becomes severely hypoxic when the perfusate is equilibrated with a normoxic gas mixture (31).

To alter the phrenic pattern to a decrementing discharge, which we have concluded is gasping (21, 30), ischemia was produced by stopping perfusion for 1 min.

Analyses of Data

Statistical analysis of data was by a nonparametric analysis of variance (Kruskal-Wallis) when more than two conditions were tested, or by t-test or Wilcoxon test when only two conditions were tested. If the Kruskal-Wallis test indicated that significant differences existed among treatments, specific preplanned comparisons of ranks between paired groups were made using P values adjusted by the Bonferroni method.

RESULTS

Preparations Having Intact Peripheral Chemoreceptors

Activities of the phrenic and mylohyoid nerves in eupnea (n = 53).

Integrated activity of the phrenic nerve was characterized by a sudden onset and then rapid rise to reach a peak level close to the end of the burst (Fig. 2). The periods of the respiratory cycle were defined by the duration of the phrenic burst (neural inspiration, Tᵢ) and the period between bursts (neural expiration, Tₑ). The sum of Tᵢ and Tₑ is the total duration of respiratory cycle (Tᵢₑ). We also defined the peak integrated phrenic discharge height (Peak Phr) and the time to reach peak height, expressed as a percentage of Tᵢ. This last variable is an index of the shape of the phrenic burst.

Activity of the mylohyoid nerve was variable between preparations and also underwent some time-dependent changes in a given preparation (Fig. 2). Yet a common and sustained char-
MYLOHYOID RESPIRATORY ACTIVITY

characteristic of all mylohyoid activities was an augmentation in discharge during neural expiration (Fig. 2, A–C). In most preparations, especially within 30 min after the start of rhythmic activities of the phrenic and mylohyoid nerves, the pattern of Fig. 2A was observed. Note that the level of discharge of the mylohyoid nerve fell in late neural expiration and remained low during neural inspiration. However, the magnitude and timing of this decline in mylohyoid discharge was highly variable.

Another common characteristic of mylohyoid discharge, which became prevalent with time during an experiment, was the presence of high amplitude bursts of short duration. These bursts could be regular, as those during late neural expiration of Fig. 2B or almost random in amplitude and timing, as those of Fig. 2C.

We quantified the activity of the mylohyoid nerve using four indexes: the peak integrated values in neural inspiration and expiration and the mean integrated values throughout neural inspiration and expiration. Baseline values for peak and mean values were defined at the end of an experiment, after rhythmic neural discharges had ceased for several minutes.

A greater level of both peak and mean integrated mylohyoid activity during neural expiration, compared with neural inspiration, was a common characteristic despite time-dependent changes in the frequency of phrenic bursts throughout an experiment. For all studies, both mean and maximal integrated mylohyoid discharge remained more than 195% of comparable values during neural inspiration despite a decline in the duration of the respiratory cycle from a mean of 3.86 to 2.51 s. However, activity during neural inspiration was still appreciable, being a minimum of three times that of baseline levels. These baseline levels were measured when rhythmic activity had ceased at the end of an experiment. In comparing changes following any perturbation, such as exposure to hypoxia or hypercapnia, variables following a perturbation were compared with the immediately preceding period in which recordings had been obtained during eupnea in hyperoxic normocapnia. Also, exclusive of ischemia-induced gasping, data were only taken from a single perturbation in any single experiment. For example, only a single level of hypercapnia or hypoxia was produced in a given preparation. Ischemia was produced only after the other perturbations had been performed.

Activities of the phrenic and mylohyoid nerves in ischemia and gasping (n = 23). Within 15 s of the termination of perfusion and onset of ischemia, the frequency of phrenic bursts declined (Fig. 3). In the interval between phrenic bursts, integrated mylohyoid activity increased greatly to levels higher than those recorded in eupnea (Figs. 3, 4). During the period of phrenic silence (apnea), the mylohyoid nerve discharged tonically. The level of this tonic discharge fell with continuing ischemia. Compared with levels during hyperoxic normocapnia, both peak and mean levels of mylohyoid discharge during neural inspiration were also significantly higher in ischemia (Fig. 4).

With continuing ischemia, gasping was induced. Gasping was evidenced by a significant increase in the rate of rise of phrenic discharge compared with that of eupnea (Fig. 3). Hence, peak phrenic discharge was achieved after 73 ± 1.4% of the burst was completed in eupnea and 38 ± 2.0% of this burst in gasping. In the interval between gasps, both peak and mean levels of integrated mylohyoid discharge remained significantly higher than those that had been recorded during neural expiration of eupnea. However, importantly, as in eupnea, this mylohyoid discharge still declined during the period of the phrenic burst of gasping (Figs. 3, 4). In gasping, peak integrated mylohyoid discharge during neural expiration averaged 211 ± 16% of the value during neural inspiration. Mean integrated mylohyoid activity in expiration averaged 169 ± 16% of the inspiratory value. Both of these indexes were significantly different (P < 0.0001) from the inspiratory level (Wilcoxon test).

Activities of the phrenic and mylohyoid nerves in hypercapnia, hypocapnia, and hypoxia. As noted in the preceding section, integrated activity of the mylohyoid nerve during both neural expiration and inspiration increased greatly during ischemia. Thus we hypothesized that similar increases in mylohyoid activity would be found in hypercapnia and hypoxia. Responses to these stimuli were individually assessed.

HYPERCAPNIA AND HYPOCAPNIA (N = 13). With a switch in the perfusate for one equilibrated with 95% O₂-5% CO₂ to a hyperoxic-hypercapnia mixture of 91% O₂-9% CO₂, peak integrated phrenic activity was not significantly changed. In contrast, integrated mylohyoid activities during both neural expiration and inspiration declined significantly and, in fact, rhythmic mylohyoid activity could not be discerned in most preparations (Fig. 5). Compared with values at normocapnia,
values of peak integrated mylohyoid discharge in hypercapnia averaged 55 and 56% during neural inspiration and expiration. Comparable values of mean integrated mylohyoid discharge during neural inspiration and expiration in hypercapnia were 71 and 56% of those in normocapnia. These declines were found in each of the five preparations examined during hypercapnia and thus represent significant declines (P < 0.05, Wilcoxon test).

Changing the gas mixture from 95% O_2-5% CO_2 to 97% O_2-3% CO_2 caused, on average, no significant change in either phrenic or mylohyoid discharges. Yet there was much variability in responses with peak integrated phrenic discharge falling in two of four preparation and peak integrated mylohyoid discharge during neural expiration rising greatly in three of four preparations.

When the perfusate was equilibrated with 100% O_2, the frequency of phrenic bursts and their peak height declined to apnea. At this time, peak and mean integrated mylohyoid discharge in both neural inspiration and expiration increased transiently in some preparations, but then also ceased.

**Hypoxia (N = 14)** Different preparations were exposed to one of three levels of reduced oxygen: 91% O_2-5% CO_2 (n = 4), 50% O_2-5% CO_2 (n = 5), 20% O_2-5% CO_2 (n = 5) (balance of nitrogen in all mixtures). The two higher levels of oxygen caused little change in integrated phrenic or mylohyoid discharges. Within 80–90 s of exposure to 20% O_2-5% CO_2, the frequency of phrenic bursts and its peak height began to decline. Concomitantly, peak and mean integrated mylohyoid discharges during neural inspiration increased in some preparations, but these increases were not consistent between preparations. Thus compared with control values in hyperoxia, peak integrated mylohyoid discharge in neural inspiration ranged from 37 to 172%; comparable values for mean integrated mylohyoid discharge ranged from 68 to 189% of control. In contrast to changes during neural inspiration, integrated mylohyoid discharge during neural expiration increased in each of the five preparations [peak = 145 ± 12% (range = 120–189%), mean = 153 ± 14% (range = 116–185%)].

Results presented in **preparations having intact peripheral chemoreceptors** demonstrated that mylohyoid discharge increased greatly in ischemia in all preparations. In hypoxia, only mylohyoid discharge during neural expiration increased in each preparation and only when the perfusate was equilibrated with 20% oxygen. We hypothesized that the augmentations in mylohyoid activity might be due in part to actions of hypoxia directly on neuronal activities within the brainstem. To assess this hypothesis, studies were conducted using preparations in which influences from the peripheral chemoreceptors had been removed by sectioning of the carotid sinus nerves and vagi.

With the perfusate equilibrated with 95% O_2-5% CO_2, discharge of the mylohyoid nerve was very similar to that which had been recorded in preparations having intact peripheral chemoreceptors. Following a switch to a perfusate equilibrated with 20% O_2-5% CO_2, the frequency and peak height of phrenic bursts fell and mylohyoid activity increased greatly in most preparations (Fig. 6). For all four preparations tested, mean changes (±SE) were as follows: phrenic 90 ± 0.4%; maximum mylohyoid inspiration, 155 ± 18%; maximum mylohyoid expiration, 141 ± 32%; mean mylohyoid inspiration, 163 ± 16%; mean mylohyoid expiration, 151 ± 26%. For mylohyoid dis-
ullary neurons is defined by the multiple pontile and medullary neuronal influences that establish eupnea. 2) An activation of the medullary pacemaker mechanisms by membrane depolarization and augmentation of the persistent sodium conductance; the latter underlies the pacemaker discharge of gasping (21, 22, 32). Implicit to this proposal for the neurogenesis of gasping is that neural influences from pons must be suppressed. Classically, such suppression has been produced by transection of the brain stem at the pontomedullary junction or through the rostral medulla (14, 15, 24, 36). Also classical is the observation that eupnea can be suppressed and gasping elicited in extreme hypoxia or ischemia (16, 26, 27). Such severe hypoxia or ischemia was envisaged both to suppress pontile neuronal activities and activate medullary pacemaker neurons that generate gasping (27, 36). Our results demonstrate that the suppression of pontile neuronal activities need not be absolute for gasping to be released.

**Maintenance of Pontile Neuronal Activities in Gasping**

During gasping, the tonic mylohyoid discharge declined during the phrenic burst. This finding that mylohyoid discharge was modulated by the respiratory cycle in gasping demonstrates that connections between medullary and pontile respiratory regions can remain functional at levels of hypoxia sufficient to release gasping. We previously reported such functional medullary-pontile projections in gasping in that some neurons of the pneumotaxic center discharge during gasps that occur in recovery from severe hypoxia (7). Evidence of axonal projections from medullary neurons having respiratory-modulated discharge patterns to pons has also been obtained (4, 23). In the context of the present report, axons of many of these medullo-pontile neurons appear to terminate in the region of the trigeminal motor nucleus (4).

Further evidence of medullo-pontile projections, albeit from neurons of unknown physiological function, has come from neuroanatomical experiments involving the transport of pseudorabies virus (see Ref. 6 for review). Projections to trigeminal motoneurons that form the mylohyoid nerve are from regions of the medulla that contain high concentrations of respiratory-modulated neuronal activities. Included in these medullary regions are the nucleus ambiguus and rostral ventrolateral medulla and also nucleus tractus solitarius. Parenthetically, projections from the region of the pontile pneumotaxic center, including the parabrachial and Kolliker-Fuse nuclei, have also been described (6).

The continuing function of pontile components of the brain stem respiratory control system in gasping provides an explanation for the observations, albeit limited, that eupnea and gasping can be recorded simultaneously. Such simultaneous recordings of the two respiratory patterns have been observed during recovery from severe hypoxia or ischemia (7, 17, 18).

Thus, in sum, results of the present and previous studies support the concept that the switch from eupnea to gasping involves primarily an activation of medullary pacemaker mechanisms for gasping. As noted above, gasping has classically been produced by a brain stem transection, which would remove all influences from pons, both of respiratory and nonrespiratory neuronal activities. Results of the present study demonstrate that such a total removal of all pontile influences...
is not necessary for activation of the medullary mechanisms that generate gasping.

**Suppression and Maintenance of Expiratory-Modulated Activities in Gasping**

In addition to changing the emphasis as to the principal mechanism responsible for eliciting gasping, namely, a release of medullary pacemaker mechanisms for gasping rather than a suppression of pontine mechanism per se, results of the present study further clarify the changes in expiratory modulated activities in gasping. A diminution or elimination of expiratory-modulated activities of spinal and cranial nerves of medullary origin has been considered as a hallmark of gasping, along with the significant augmentation in the rate of rise of phrenic activity (9, 26). The suppression of expiratory activities in gasping has been shown to reflect two additive processes. 1) A switch in the control of expiratory activities from the pontomedullary circuit for eupnea to the medullary mechanisms for gasping. 2) An inhibitory influence of hypoxia on activities of brain stem respiratory neurons, which is more profound for those having expiratory-modulated activities (7, 13). The most direct evidence that a switch to medullary mechanisms for gasping alone causes depressions of expiratory activities is shown in studies in which gasping has been produced by elicitation of the “aspiration reflex.” Hence, mechanical stimulation of the pharynx causes a suppression of eupneic phrenic discharge and recruitment of gasps (9). This reflex can be elicited against a background of hypoxia. On eliciting this reflex, expiratory-modulated activities of spinal nerve are depressed, whereas expiratory activities of the recurrent laryngeal nerve and branches thereof are unchanged or augmented. With an elicitation of gasping in the same preparation in severe hypoxia, expiratory activities of both spinal and cranial nerves are depressed (8). Thus brain stem hypoxia alone contributes to the suppression of expiratory modulated activities in gasping.

Results of the present study demonstrate that a suppression of expiratory activity does not always accompany gasping. While such a suppression does seem ubiquitous in gasping produced by brain stem transections, severe hypoxia can suppress or augment expiratory activities of different nerves in a highly selective manner. Thus, separate from releasing medullary mechanisms for gasping, brain stem hypoxia also inhibits or augments expiratory discharges. These separate influences of brain stem hypoxia are clearly demonstrated in a recent paper using, as herein, the in situ preparation of the rat (1). Following a brain stem transection that eliminated the pons and rostral medulla, phrenic discharge changed to a gasping pattern (see also Ref. 24) and expiratory activities of the vagus and abdominal nerve were eliminated. When different preparations were exposed to severe hypoxia, again phrenic discharge changed to a gasping pattern and expiratory vagal discharge was eliminated. Yet, in gasping, an appreciable discharge of the abdominal nerve was recorded, albeit limited to the very beginning and end of neural expiration.

**Inhibition of Mylohyoid Discharge in Hypercapnia**

The inhibition of mylohyoid discharge in hypercapnia was totally unexpected and is inexplicable. The unexpected nature of this finding is due to the directly opposite change that is seen in other species; for example, mylohyoid discharge increases greatly during hypercapnia in cats (28). To our knowledge, such an inhibition of mylohyoid discharge is unique among cranial and spinal nerves, except in extreme hypercapnia in which respiratory activity is generally inhibited. This inhibition does not appear to reflect a general response of the in situ preparation of the rat as multiple studies document that, as in vivo, expiratory activities of cranial and spinal nerves of the in situ preparation increase in hypercapnia (1, 13). However, the ventilatory response to carbon dioxide is very steep in the in situ preparation, with little or no increase in peak phrenic discharge in extreme hypercapnia (1, 30). Hence, such extreme hypercapnia might represent a level that is depressive for mylohyoid discharge.

In the context of responses to alterations in carbon dioxide in the perfusate, we found that there was no statistically significant decline in peak integrated phrenic height with a reduction in CO₂ from 5 to 3%. This lack of a statistically significant decline in hypocapnia reflects variability in the four preparations examined. Such variability was shown in a more detailed evaluation of responses to various levels of hypocapnia (see Figs. 2 and 3 in Ref. 30). Thus, stated differently, in the in situ preparation, the apneic threshold differs in different preparations and, above the apneic threshold, peak integrated phrenic discharge increases with further elevations in CO₂.

**Augmentation of Expiratory Mylohyoid Discharge by Brain Stem Hypoxia**

The augmentation of expiratory mylohyoid discharge in hypoxia that was recorded was unexpected especially as hypoxia appears to involve actions directly on the brain stem. The conclusion of actions of hypoxia on the brain stem is supported by two findings. 1) Expiratory mylohyoid discharge increased in hypoxia in all four preparations tested following denervation of the peripheral chemoreceptors. 2) Following these denervations, similar increases in expiratory mylohyoid activity were recorded in ischemia. Although ischemia would induce both hypoxia and hypercapnia, hypercapnia results in an inhibition of integrated mylohyoid discharge, which we did not see during ischemia. When the hypoxic exposures to 20% O₂ and the ischemic tests are considered together, an increase in peak expiratory mylohyoid activity was observed in 26 of 28 trials and in mean expiratory mylohyoid activity in 28 of 28 trials in preparations having intact carotid sinus nerves and vagi. This sum of results provide strong support for the conclusion that hypoxia, whether normocapnic or hypercapnic, enhances expiratory mylohyoid activity.

Since the work of Heymans (see Ref. 12 for review), it has been recognized that the ventilatory response to hypoxia represents the net result of two processes. The first is an augmentation in respiratory activity through activation of mechanisms within the peripheral chemoreceptors. The second is a depression of respiration by actions of hypoxia directly on the brain stem. While the primary influence of hypoxia on the brain stem is inhibitory, augmentations in neuronal activities have also been found. For the respiratory system, a consistent excitatory influence of brain stem hypoxia on medullary mechanisms for gasping has been reported. Borison and Brodie (5) were the first to report that administrations of sodium cyanide augmented the frequency of gasping after denervation of the peripheral chemoreceptors. In a similar preparation, we confirmed these findings as hypoxia increased the frequency of
gasing that was produced by brain stem transections (25). In addition to gasing, brain stem hypoxia, acting at a number of sites, has been reported to cause increases in cardiovascular and/or respiratory activity (11, 20).

Functional Implications

As for other cranial nerves that may have respiratory-modulated discharges, the mylohyoid nerve has activities that are also linked to other behaviors, especially swallowing (10, 19, 33). Thus the linkage of mylohyoid discharge with the breathing cycle is not absolute. This characterization of mylohyoid discharge, including its unique response to hypercapnia and hypoxia, further supports our conclusion that the attempt to judge the type of breathing, specifically eupnea or gasping, from the discharge of any single cranial nerve is, at best, imprecise (29).

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DISCLOSURES

No conflicts of interest are declared by the authors.

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