

# Influence of levels of carbon dioxide and oxygen upon gasping in perfused rat preparation

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## Abstract

In vivo, the augmenting pattern of integrated phrenic nerve discharge of eupnea is altered to the decremting pattern of gasping in severe hypoxia or ischaemia. Identical alterations in phrenic discharge are found in perfused in situ preparations of the juvenile rat. In this preparation, gasping was produced by equilibration of the perfusate with various levels of carbon dioxide and oxygen. The duration of the phrenic burst, the interval between bursts and the burst amplitude were not significantly different following equilibration with 21–6%O<sub>2</sub> at 5% CO<sub>2</sub> or with 0–9% CO<sub>2</sub> at 6% O<sub>2</sub>, with the exception that the burst amplitude was significantly greater in hypercapnic-hypoxia (9% CO<sub>2</sub> at 6% O<sub>2</sub>). It is proposed that hypoxia-induced gasping results from the release of an endogenous pacemaker activity of rostral medullary neurons. This release is caused by cellular mechanisms that change the balance between membrane ionic currents. Moreover, these cellular mechanisms may be explicitly induced by alterations in the ionic and metabolic homeostasis. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Eupnea and gasping represent two patterns of automatic ventilatory activity, which differ in a multiple aspects. A fundamental difference is that in eupnea, activity of the phrenic nerve increases in a ‘ramp-like’ manner whereas, in gasping, phrenic discharges have a ‘decremting’ pattern (St.-John, 1996, 1998).

Responses to hypercapnia and hypoxia likewise differ in eupnea and gasping. During eupnea in decerebrate animals, hypercapnia causes an increase in both amplitude of integrated phrenic discharges and respiratory frequency. Ventilatory activity declines in hypocapnia and finally ceases at an ‘apneic threshold’. Hypoxia also causes augmentations in amplitude and frequency of phrenic bursts. As opposed to these changes, during gasping resulting from brainstem transections in vivo, hypercapnia causes no systematic alterations in phrenic activity. In addition, no ‘apneic threshold’ for gasping can be demonstrated in

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hypocapnia. Hypoxia does cause a transient augmentation in frequency, but not in amplitude of gasps. However, these responses to hypoxia appear to represent direct actions upon medullary neurons, as responses are not altered by denervation of the peripheral chemoreceptors (St.-John, 1990, 1996, 1998).

An *in situ* preparation of the juvenile rat exhibits patterns of automatic ventilatory activity which are very similar to eupnea and gasping *in vivo* (St.-John and Paton, 2000). In this preparation, we have recently found that a blockade of inhibitory synaptic transmission within the brainstem by administrations of bicuculline, picrotoxin or strychnine severely disrupts eupnea but causes little or no alteration in gasping (St.-John and Paton, 2001). This finding, and others described above, have led to the concept that eupnea and gasping are produced by different neuronal mechanisms (St.-John, 1996, 1998; Fukuda, 2000). The eupneic rhythm results from mutual interactions between functionally distinct neural populations in a ponto-medullary circuit, whereas gasping is driven by endogenous bursting activity of conditional pacemaker neurons in the rostral medulla. During eupnea, this pacemaker activity is suppressed, and the rostral medullary neurons function as a part of the ponto-medullary circuit (St.-John, 1996; Rybak et al., 2001; St.-John and Paton, 2001).

The pacemaker/bursting activity of rostral medullary neurons may release and dominate rhythmogenesis under a number of experimental conditions. Since the initial characterization by Lumsden (1923), it has been recognized that gasping typically follows a brainstem transection at the ponto-medullary junction or exposure to severe hypoxia or ischaemia. The neurogenesis of gasping during hypoxia appears to result from changing the balance between ionic currents in some neurons, as well as by alteration of ionic/metabolic homeostasis in the neuronal environment. Hence, in addition to hypoxia *per se*, gasping *in vivo* can be produced in normoxia or hyperoxia by exposure to carbon monoxide (Melton et al., 1991, 1996; Zhou et al., 1991) or injections of cyanide, either systemically or into the rostral medulla (Brodie and Borison, 1956;

Solomon et al., 2000). Note that the presence of cyanide or carbon monoxide would not cause a reduction in partial pressures of oxygen in the medulla.

The present studies were undertaken in order to characterize further the brainstem environment which allows for a release of medullary mechanisms for gasping. Using the perfused preparation of the juvenile rat (St.-John and Paton, 2000), the influence of various levels of oxygen and carbon dioxide upon the elicitation of gasping has been evaluated. The results of this evaluation provide insights into the mechanisms whereby hypoxia alters normal functioning of the ponto-medullary neuronal circuit underlying eupnea and releases the medullary pacemaker-driven mechanisms of gasping.

## 2. Methods

### 2.1. The preparation

Eleven juvenile rats (80–170 g) were used. The preparation has been described in detail previously (Paton, 1996a,b; St.-John and Paton, 2000). Briefly, rats are anesthetized with halothane and decerebrated at a precollicular level. Prior to or immediately following decerebration, body temperature is reduced by covering the body with crushed ice or immersion in artificial cerebrospinal fluid, which is cooled to a temperature of less than 10 °C. The portion of the body caudal to the diaphragm is removed. The phrenic nerves are sectioned. A catheter is inserted into the descending aorta (double lumen, size 3.5 or 4.0 French), advanced rostrally and tied in place. Any ice which surrounded the body has been removed by this time. Perfusion is then commenced, with perfusion pressure being increased gradually until activity of the phrenic nerve returns with a ‘ramp-like pattern’. This activity is monitored by a bipolar metal or glass ‘suction’ electrode, amplified, filtered (0.6–6.0 kHz), integrated (50 ms time constant) and recorded.

Gallamine triethiodide (0.5 mg ml<sup>-1</sup>) is added to the perfusate to eliminate motor movements.

The temperature of the perfusate, as it enters the aorta, is maintained at 30–31 °C.

The perfusate contains the following in distilled water, magnesium sulfate ( $\text{MgSO}_4$ , 1.25 mM), potassium phosphate ( $\text{KH}_2\text{PO}_4$ , 1.25 mM), potassium chloride (KCl, 5.0 mM), sodium bicarbonate ( $\text{NaHCO}_3$ , 25 mM), sodium chloride (NaCl, 125 mM), calcium chloride ( $\text{CaCl}_2$ , 2.5 mM), dextrose (10 mM), Ficoll 70 (0.1785 mM). Under control conditions, the perfusate is equilibrated with 95%  $\text{O}_2$ –5%  $\text{CO}_2$ . Measured at 31 °C, the pH of the perfusate is 7.38.

From a reservoir, the perfusate passes through a roller pump, a filter (Millipore 45  $\mu\text{M}$ ), two ‘bubble traps’ and then the cannula in the aorta. Perfusate leaks from the numerous sectioned vessels and collects in a reservoir surrounding the animal. The perfusate is recirculated and re-equilibrated with 95%  $\text{O}_2$ –5%  $\text{CO}_2$ .

## 2.2. Alterations from eupnea to gasping

As noted above, control conditions were equilibration of the perfusate with 95%  $\text{O}_2$ –5%  $\text{CO}_2$ . In order to assess the influence of various levels of  $\text{O}_2$  and  $\text{CO}_2$  upon gasping, perfusate in a second reservoir was equilibrated with a variety of gas mixtures. Perfusate from this second reservoir was delivered to the preparation until gasps were recorded after which the ‘control’ perfusate was reintroduced. A minimum of 10 min was allowed for recovery of the eupneic pattern.

To assess the influence of changes in oxygen, the following mixtures were used, 21%  $\text{O}_2$ –5%  $\text{CO}_2$ ; 11%  $\text{O}_2$ –5%  $\text{CO}_2$ ; 6%  $\text{O}_2$ –5%  $\text{CO}_2$ . To assess the influence of changes in carbon dioxide, the mixtures were, 6%  $\text{O}_2$ –5%  $\text{CO}_2$ ; 6%  $\text{O}_2$ –9%  $\text{CO}_2$ ; 6%  $\text{O}_2$ –2%  $\text{CO}_2$ ; 6%  $\text{O}_2$ –0%  $\text{CO}_2$ . In some trials involving hypocapnia, a perfusate, which was equilibrated with a hyperoxic–hypocapnic mixture, was infused during eupnea. This perfusate was then replaced with one equilibrated with hypoxia, at the same level of hypocapnia.

## 2.3. Analysis of data

Integrated activity of the phrenic nerve was quantified as to the duration of the burst

(‘neural inspiration’,  $T_I$ ), the interval between bursts (‘neural expiration’,  $T_E$ ), the total duration of the respiratory cycle ( $T_{TOT}$  and its reciprocal) and the burst amplitude (peak height). Also defined was the time after the onset of hypoxia at which the first gasp occurred. Statistical evaluations were by the non-parametric Wilcoxon test. Probabilities less than 0.05 were considered as significant.

## 3. Results

### 3.1. Characteristics of the gasp

The pattern of the gasp in the perfused juvenile rat preparation has been described in detail in previous studies (St.-John and Paton, 2000). Gasping replaced eupnea within approximately 60–120 sec after a change in perfusate from one equilibrated with a hyperoxic gas mixture to another equilibrated with a hypoxic mixture (Fig. 1). As opposed to the ramp-like rise of eupnea, integrated phrenic activity in gasping had a ‘square-wave’ or decremting pattern, with peak activity being reached soon after onset. Except for recordings during hypocapnia, as detailed below, a minimum of five gasps were recorded and analyzed.

### 3.2. Responses to alterations in oxygen

In six of seven preparations, the eupneic pattern was replaced by gasping when the preparation was perfused with a solution equilibrated with 21%  $\text{O}_2$  and 5%  $\text{CO}_2$  (Fig. 1). For the other preparation, the eupneic phrenic burst gradually declined and ultimately ceased; no gasping was recorded. In all preparations, gasping was elicited when perfusates were used which were equilibrated with 11%  $\text{O}_2$ –5%  $\text{CO}_2$  or 6%  $\text{O}_2$ –5%  $\text{CO}_2$ . Variables of phrenic activity in gasping were not significantly different for evaluations at any of the levels of  $\text{O}_2$  (Fig. 2). The time between the switch to the normoxic or hypoxic perfusate and the first gasp was not significantly different with any of the perfusates.

### 3.3. Responses to alterations in carbon dioxide

Control conditions during gasping were considered as variables recorded with the perfusate equilibrated with 6% O<sub>2</sub> and 5% CO<sub>2</sub> (Fig. 3). Comparison of these variables with those recorded following exposure to 6% O<sub>2</sub>–9% CO<sub>2</sub> revealed only a small increase in amplitude of phrenic bursts (Figs. 3 and 4). However, in three preparations, the burst amplitude fell in hypercapnia. In six of eleven preparations, the frequency of gasping was higher in hypercapnia, this increase was due to reductions in the interval between bursts.

Gasping was elicited following exposure to perfusates equilibrated with 6% O<sub>2</sub> and 2–0% CO<sub>2</sub> (Fig. 3). This elicitation of gasping was found even when the preparation had been perfused with a solution which had been equilibrated with 98–100% O<sub>2</sub> and 2–0% CO<sub>2</sub> prior to perfusion with one having 6% O<sub>2</sub>. Upon introduction of the hyperoxic–hypocapnic perfusate, the amplitude of the eupneic phrenic bursts declined and phasic activity was eliminated. This phasic activity returned with a gasping pattern upon introduction of the hypoxic–hypocapnic perfusate.

Compared with values recorded at hypoxic–normocapnia, variables of phrenic activity were not significantly different at hypoxic–hypocapnia (Fig. 4). However, as opposed to examinations at normo- or hypercapnia, gasping was not sustained in hypocapnia. Hence, more than three gasps could only be elicited in only two of six preparations. Even in these two preparations, peak amplitude of gasps fell after two or three gasps (Fig. 3) and apnea resulted. For the four other preparations, a sustained apnea followed the three gasps.

### 4. Discussion

The major conclusion of this study is that both the neurogenesis and regulation of gasping are primarily dependent upon the local chemical environment of the rostral medulla. Results concerning the influence of various levels of oxygen and carbon dioxide upon gasping provide insights into the mechanisms by which a pacemaker-based mechanism for gasping is activated in the rostral medulla to overcome the network-based pontomedullary rhythmogenesis mechanism for eupnea.

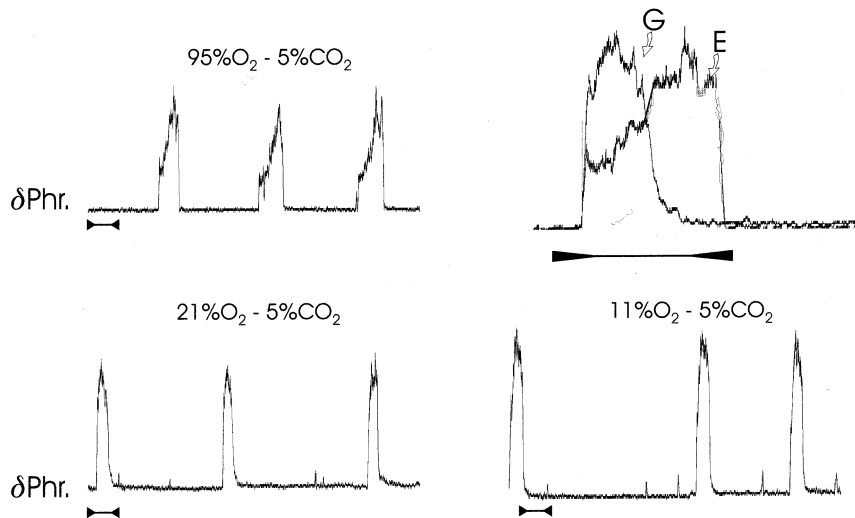


Fig. 1. Induction of gasping by reduction in levels of oxygen. In upper panel, integrated activity of the phrenic nerve is shown during eupnea, with perfusate equilibrated with 95% oxygen and 5% carbon dioxide. Lower panels show integrated activity of the phrenic nerve during gasping, established at the designated levels of oxygen. A single integrated phrenic cycle in eupnea (E) and gasping (G) is also shown. Note difference in rate of rise of phrenic activity. Time bars each represent 2.0 sec.

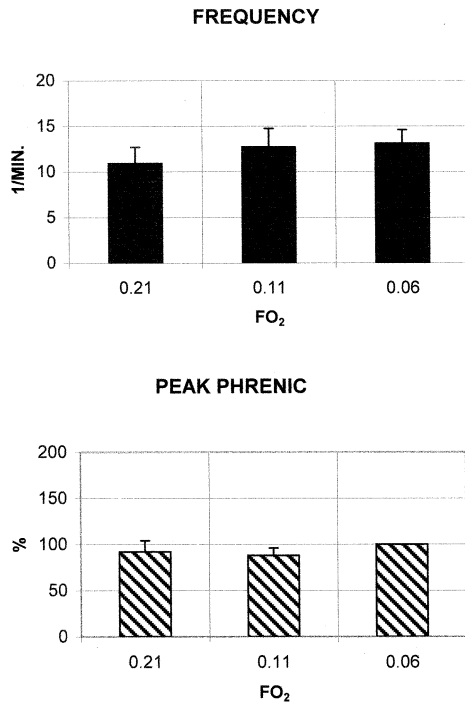


Fig. 2. Influence of levels of oxygen upon variables of gasping. Panels show mean values (+ standard error (S.E.)) for frequency and amplitude of integrated phrenic activity in gasping at designated fractional concentrations of oxygen (FO<sub>2</sub>). All responses were evaluated at fractional concentration of carbon dioxide of 0.05. Values for peak phrenic amplitude have been normalized as a percentage of those recorded at FO<sub>2</sub> of 0.06.

A striking result of this study was that gasping could be induced, and had identical characteristics, when the perfusate was equilibrated with oxygen concentrations varying from 21 to 6%. Inherent in this observation is the conclusion that the switch from eupnea to gasping cannot reflect solely the explicit activation of a ‘central oxygen detector’ in the medulla. Rather, a primary mechanism in this activation must be an alteration in the chemical content of the extracellular and/or intracellular environment, which in turn influences the firing behavior of some limited population of medullary neurons.

Parenthetically, since the sole source of oxygen for the perfused preparation of the juvenile rat is that in solution, it is perhaps expected that eupnea could not be maintained at normoxia. While all levels of oxygenation were not evaluated, yet eup-

nea is maintained if the perfusate is equilibrated with 50% oxygen (unpublished observation). Moreover, as recently shown directly by Deutschmann et al. (2000), Wilson et al. (2001) in the perfused preparations of the neonatal and juvenile rats, respectively, levels of oxygen within the brainstem are hyperoxic when the perfusate is equilibrated with 95% O<sub>2</sub>–5% CO<sub>2</sub>.

There is much experimental evidence that the neurogenesis of gasping is dependent upon neuronal activities in the rostral medullary ‘gasping center–preBotzinger complex’ (see St.-John, 1996, 1998 for review). The question obviously arises as to how hypoxia induces or releases endogenous pacemaker/bursting firing behavior of these rostral medullary neurons. One primary component in the release of pacemaker behavior is the suppression of inhibitory synaptic transmission within the pontomedullary respiratory network (Richter et al., 1991). In this context, we have recently reported that a blockade of inhibitory synaptic transmission severely disrupts eupnea but not gasping in the perfused juvenile rat preparation. This disruption of eupnea was not such that the pattern was changed to gasping (St.-John and Paton, 2001). However, a switch from eupnea to gasping did occur when this blockade of inhibitory transmission was combined with an augmentation in the extracellular potassium concentration and a blockade of potassium channels by 4-aminopyridine (St.-John et al., 2001). Brainstem hypoxia may itself induce such changes in extracellular potassium and in the functioning of potassium channels.

The extracellular concentration of potassium is augmented in hypoxia (Melton et al., 1991, 1996). This augmentation occurs immediately prior to and immediately after the onset of gasping (Melton et al., 1991) and probably results from the increased neuronal activity.

Concerning ion channels, hypoxia suppresses several types of potassium channels and activates sodium channels, especially the persistent sodium channels, of neurons in many regions (e.g. Jiang and Haddad, 1994; Lopez-Barneo, 1996; Hammarstrom and Gage, 1998; Thompson and Nurse, 1998; Gebhardt and Heinemann, 1999; Kawai et al., 1999; Liu et al., 1999; Horn and Waldrop,

2000; Prabhakar, 2000; Lopez-Barneo et al., 2001). The exact mechanisms intermediating the hypoxia-induced changes in the functioning of ionic channels and other intrinsic neuronal properties are not well defined. These mechanisms may involve signaling pathways and second messenger systems at the intracellular level. Moreover, hypoxia may modify channel conductances and neuronal firing properties through multiple cellular/intracellular mechanisms (Lopez-Barneo et al., 2001).

The hypoxia-induced processes, such as alteration of the ionic/metabolic extracellular environment, modulation of the intrinsic neuronal properties, and suppression of synaptic inhibition, cannot of course be limited to the region for neurogenesis of gasping in the rostral ventrolateral medulla. Rather, hypoxia-induced processes would be altered in many regions of the brainstem and, in intact animals, in the rest of the brain as well. However, recent studies have demonstrated that neurons in the rostral ventrolateral medulla have a high intrinsic chemosensitivity to hypoxia (Kawai et al., 1999; Solomon et al., 2000). The question of what intrinsic properties of these neurons define their special role in genesis of

pacemaker driven gasping-like oscillations is undefined. One possible property that distinguishes these neurons from other brainstem neurons may be potassium channels with higher sensitivity to oxygen (Solomon et al., 2000; Rybak et al., 2001).

Missing from the above consideration is the role of carbon dioxide and pH in the elicitation of gasping. Since gasping can still be elicited in severe hypocapnia, it would appear that the initial hypoxia-induced change in neuronal function is independent of the concentration of hydrogen ion. The observation that gasping only persisted for a brief interval in hypocapnia may reflect the influence of hydrogen ion on membrane channels and ion pumps (Moody, 1984). In this context, the one variable of gasping which was influenced by the level of carbon dioxide and/or oxygen was an augmentation in amplitude of gasps in severe hypercapnia. This observation may imply that central chemoreceptor mechanisms can alter gasping. However, these responses to hypercapnia differ from findings from en bloc brainstem–spinal cord preparations, which have been concluded to be exhibiting gasping (St.-John, 1996; Remmers, 1998). In these preparations, hypercapnia and/or

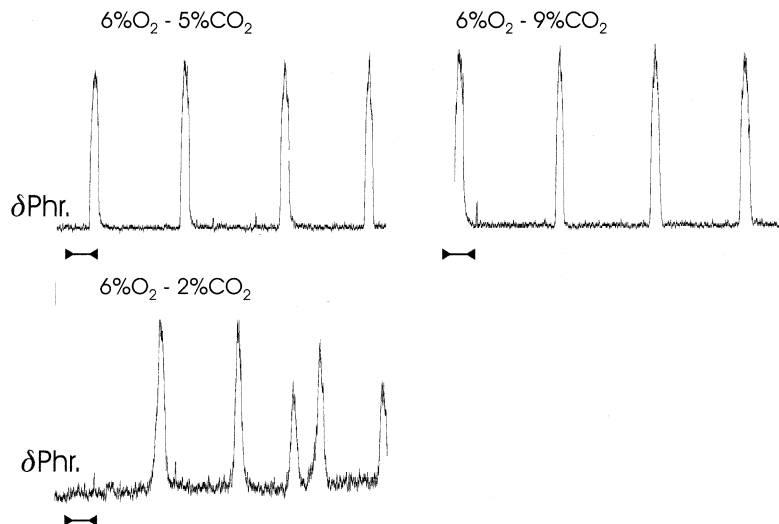


Fig. 3. Gasping in hypercapnia and hypocapnia. Panels show integrated activity of the phrenic nerve at the designated levels of oxygen and carbon dioxide. Note persistence of gasping in hypocapnia. Time bars each represent 2.0 sec.

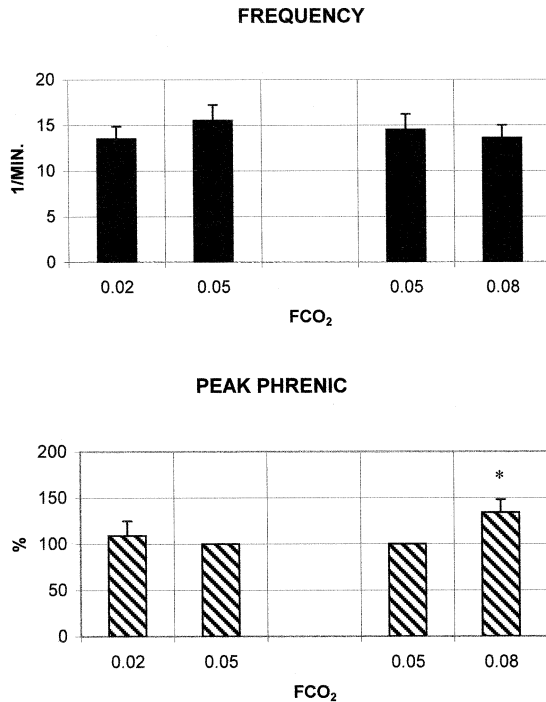


Fig. 4. Influence of levels of carbon dioxide upon variables of hypoxia-induced gasping. Panels show mean values (+ S.E.) for frequency and amplitude of integrated phrenic activity in gasping at designated fractional concentrations of carbon dioxide (FCO<sub>2</sub>). All responses were evaluated at fractional concentration of oxygen of 0.06. Note that not all preparations were exposed to both hypocapnia and hypercapnia. Controls for each group are the same preparations which were examined at normocapnia. \* $P < 0.05$  compared with value at normocapnia.

acidosis causes an increase in frequency, but not amplitude, of rhythmic bursts (see Ballantyne and Scheid, 2000 for review). One possible reason for this difference may be the environment of the central chemoreceptor mechanisms. Hence, oxygenation of the *in vitro* preparation varies greatly throughout the tissue, being adequate for neuronal function close to the surface and then falling sharply (see discussions in St.-John, 1996; Remmers, 1998). In contrast, tissue levels of oxygen should be more consistent, though by design hypoxic, in the perfused preparation exhibiting gasping. Thus, the level of oxygenation of 'chemoreceptor mechanisms' especially those in the region of the ventrolateral medulla, would be expected to differ in the two

types of preparations. Further confounding responses to hypercapnia/acidosis are results from medullary slice preparations which have a similar rhythmic output to *en bloc in vitro* preparations. Hypercapnia/acidosis is reported to cause an increase in the frequency of these rhythmic bursts in some studies but no change in frequency in other reports (see Peever et al., 1999 for review).

As opposed to hypercapnia, hypoxia would appear directly to increase excitability of neurons within the rostral medulla 'gasp center'–'pre-Botzinger complex'. Thus, it is well recognized that hypoxia results in an augmentation, and then a decline, in the rhythmic bursts of *en bloc* and slice *in vitro* preparations (e.g. Ballanyi et al., 2000; Thoby-Brisson and Ramirez, 2000). An identical pattern of change is observed *in vivo* in steady-state gasping, resulting from brainstem transections at the ponto–medullary junction (St. John and Knuth, 1981; St.-John, 1996). We believe it most probable that this transient increase in frequency results from a transient hypoxia-induced increase in the concentration of extracellular potassium.

In summary, results of the present study provide additional insights into the mechanisms by which hypoxia induces a reconfiguration of the brainstem ventilatory control system from the pontomedullary neuronal circuit which generates eupnea to the medullary pacemaker system which generates the gasp. The influence of central chemoreceptors mechanism is defining the magnitude and frequency of phrenic bursts is much reduced in gasping as compared with eupnea. Such a reduction is consistent with the concept that the magnitude and frequency of gasps are primarily defined by the local environment of the rostral medullary 'pre-Botzinger complex–gasp center'.

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