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Eupnea of *in situ* rats persists following blockers of *in vitro* pacemaker burster activities

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Abstract

Two groups of intrinsically bursting neurons, linked to respiration, have been identified using *in vitro* medullary slice preparations. One group is dependent upon a calcium-activated nonspecific cationic current that is blocked by flufenamic acid. This group is hypothesized as essential for eupnea, but not gasping. The second group is dependent upon conductance through persistent sodium channels that is blocked by riluzole. This group is proposed to underlie both eupnea and gasping. In the decerebrate *in situ* preparation of the juvenile rat, flufenamic acid caused an increase in frequency and a decrease in peak level of the phrenic and vagus nerve activities in both eupnea and gasping. Similar changes in eupnea followed the simultaneous blockades by flufenamic acid and riluzole. However, gasping was eliminated. These results do not support the hypothesis that conductances through either persistent sodium channels or calcium-activated nonspecific cationic channels are essential for the neurogenesis of eupnea. However, gasping does depend upon a conductance through persistent sodium channels.

1. Introduction

The role of intrinsically bursting neurons in the neurogenesis of normal, eupneic breathing is unresolved and controversial (Paton and St.-John, 2007; Ramirez and Garcia, 2007). One group of burster neurons is dependent upon conductance through persistent sodium channels. Blockers of this conductance eliminate some *in vitro* rhythms including one considered to be the basis of eupnea. However, these blockers do not interrupt the eupneic rhythm of *in situ* rat preparations having an intact pontomedullary brainstem or of *in vivo* neonatal mice or adult rats (Paton *et al.*, 2006; St.-John *et al.*, 2007). Gasping in all preparations, including *in vitro*, *in situ* and *in vivo*, is eliminated by these blockers (see Paton and St.-John, 2007 for review).

A second group of intrinsic bursters is dependent upon a calcium-activated nonspecific cationic current (Thoby-Brisson and Ramirez, 2001). Blockers of this current have been reported to suppress an eupneic *in vitro* rhythm, but not a gasping rhythm. This eupneic-type rhythm is not eliminated by blockers of these calcium-dependent current alone, rather simultaneous application of blockers of the conductance through the persistent sodium channel is also required (Ramirez and Garcia, 2007).

In an intact, unanaesthetized, spontaneously breathing neonatal mouse preparation, it is also reported that blockade of conductances through both persistent sodium and calcium channels

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eliminates eupnea, whereas a blockade of the former alone eliminates gasping (Pena and Aguilera, 2007). However, the changes in ventilatory activity of spontaneously breathing preparations might reflect an alteration in multiple mechanisms, including those secondary to an alteration in cardiovascular function. The *in situ* preparation of the perfused juvenile rat is not dependent upon cardiovascular function for its viability and moreover can exhibit the various patterns of automatic ventilatory activity, including eupnea and gasping (St.-John and Paton, 2000; Paton *et al.*, 2006). Using this preparation, we have undertaken further studies to assess the influence of blockers of the calcium-activated nonspecific cationic current and the persistent sodium current upon both eupnea and gasping. Based upon finding from *in vitro* preparations, blockers of the calcium-activated non-specific cationic current should preferentially suppress eupnea, but not gasping whereas a combination of these blockers with those for persistent sodium current should eliminate eupnea and gasping simultaneously.

2. Methods

2.1 Experimental preparations

Fifty-one perfused preparations of the juvenile rat were used. The preparation was identical to that described previously (St.-John and Paton, 2000; Paton *et al.*, 2006), with surgical procedures being performed under deep enflurane anaesthesia. Anaesthesia was discontinued following pre-collicular decerebration.

The descending aorta was cannulated and perfusion was commenced. The constituents of the perfusate were identical to those described previously (St.-John and Paton, 2000). Gallamine triethiodide was added to block neuromuscular transmission. The temperature of the perfusate as it entered the aorta was 31° C. Efferent activity of the phrenic nerve was recorded in all preparations and of the vagus nerve in twenty-five preparations.

2.2 Antagonists

To block calcium activated, nonspecific cation current, flufenamic acid (*N*-(3-[Trifluoromethyl]phenyl)anthranilic acid, Sigma) was added to the perfusate. Flufenamic acid was prepared as a 2.5 mM solution in equal volumes of ethanol and (2-hydroxypropyl)- β -cyclodextrin solution (45% w/v, Sigma), which prevented precipitation in the perfusate. To block conductance through persistent sodium channels, riluzole (2-Amino-6-(trifluoromethoxy)benzothiazole, Sigma) was used. Riluzole was dissolved in dimethyl sulfoxide.

2.3 Experimental Protocols

A minimum of thirty minutes was taken following the start of perfusion before neural activities were recorded in hyperoxic normocapnia (95% O₂–5% CO₂) during eupnea. In control experiments using twenty-five rats, neural activities were then recorded in hypoxic-hypercapnia (5.5–6.5% O₂–7–9% CO₂) during gasping. In separate preparations, flufenamic acid (nineteen rats) or flufenamic acid and riluzole in combination (seven rats) were administered during initial recordings in eupnea and then, ten minutes thereafter, hypoxic hypercapnia was introduced in an attempt to elicit gasping. Any single preparation only received one concentration of drug or combination of drugs.

Statistical evaluations were made by Wilcoxon tests or regression analyses.

3 Results

3.1 Eupnea – Gasping Comparison

With the perfusate equilibrated with a hyperoxic-normocapnic gas mixture (95% O₂–5% CO₂), integrated phrenic discharge had the incrementing pattern of eupnea, as described previously (St.-John and Paton, 2000; Paton *et al.*, 2006). Efferent vagal activity had a burst of activity during the period of the phrenic burst, and a larger burst during the early portion of neural expiration, at the termination of the phrenic discharge (Figs. 1,2). Changes in integrated activities of the phrenic and vagus nerves with exposure to hypoxic – hypercapnia were as described in multiple publications previously (e.g., St.-John and Paton, 2000; Paton *et al.*, 2006). Thus, the pattern of phrenic discharge changed from one in which peak activity was reached in the last half of the burst (63 ± 3.0 % of neural inspiration) to one in which peak activity was achieved earlier (45 ± 2.0 %). These changes are typical of gasping. The frequency of gasps was not significantly different from that of eupnea (96.5 ± 7.6 % of eupnea), nor was the peak integrated phrenic level (103 ± 4.5 % of eupnea). In gasping, peak inspiratory vagal discharge was as eupnea (106 ± 4.6 %), whereas peak expiratory vagal discharge had declined (82 ± 7.6% of eupnea).

3.2. Changes in eupnea and gasping following flufanemic acid

Following administrations of 10 – 50 µM of flufanemic acid, the respiratory frequency increased (Fig. 1). However, the increase in frequency was not significantly related to the concentration of flufanemic acid (regression analysis) and, moreover, was within the range of changes observed over the same time course in preparations that had received no flufanemic acid. Peak level of phrenic discharge did decline significantly with increasing concentrations of flufanemic acid ($P < 0.001$, regression analysis). For preparations in which efferent vagal activity was recorded, peak activities in neural inspiration and expiration fell in parallel with changes in peak phrenic activity (Fig. 1). In some preparations (e.g., Fig. 1), expiratory vagal activity was only observed in a limited proportion of respiratory cycles following flufanemic acid. Importantly, even in experiments in which phrenic and vagal discharges were barely discernible, rhythmic discharges were still observed.

In preparations that had received flufanemic acid, exposure to hypoxic hypercapnia resulted in an alteration in the patterns of integrated phrenic and vagal activities to those typical of gasping (Fig. 1). Multiple gasps were recorded except for three preparations in which only one or no gasps were recorded after 15, 20 and 50 µM of flufanemic acid. For the other preparations, the frequency of phrenic bursts in gasping increased and peak phrenic height declined with increasing concentrations of flufanemic acid.

3.3 Changes in eupnea and gasping following flufanemic acid and riluzole

Flufanemic acid and riluzole were added simultaneously to the perfusate of seven rats. The concentration of riluzole was 10 µM in six preparations and 5 µM in the other. These concentrations of riluzole were similar to those which we have previously reported as reducing the number of gasps or eliminating gasping entirely (Paton *et al.*, 2006). The concentrations of flufanemic acid were 30 µM in four rats and 40 µM in the others. Compared to control preparations that received no drugs, the level of phrenic bursts fell significantly with the administrations of flufanemic acid and riluzole ($P < 0.01$, regression analysis) (Fig. 2). The frequency of phrenic bursts increased but this increase was not related to the concentrations of drugs (Fig. 2). Peak activities of vagal inspiratory and expiratory activities also fell along with the reductions in peak phrenic activity.

Upon exposure to hypoxic hypercapnia, gasping could not be discerned in four preparations (Fig. 2) or was limited to bursts of extremely small amplitude (three preparations).

4. Discussion

4.1 Overall Conclusions

Results herein do not support the hypothesis that intrinsic medullary bursters which are dependent upon a calcium-activated nonspecific cationic current play a fundamental role in the neurogenesis of eupnea, but not gasping (Pena *et al.*, 2004; Pena and Aguileta, 2007; Ramirez and Garcia, 2007). Such a differential role in eupnea and gasping would imply that blockers of this current should preferentially suppress eupneic ventilatory activity. A preferential suppression was not found with flufanemic acid causing equivalent suppressions of phrenic and vagal activity in both eupnea and gasping. These results moreover do not confirm the observations from *in vitro* preparations that a simultaneous blockade of conductances through both persistent sodium and calcium channels eliminates eupnea (Pena *et al.*, 2004; Pena and Aguileta, 2007; Ramirez and Garcia, 2007). This difference in findings obtained *in vitro* and *in situ* emphasizes that the relationship between *in vitro* rhythms of en bloc and/or medullary slice preparations and those of preparations having an intact pontomedullary brainstem, which we consider akin to eupnea, remains nebulous (Paton and St.-John, 2007). Finally, the marked reduction in phrenic discharge amplitude which we observed following administrations of flufanemic acid alone or in combination with riluzole implies that breathing would be seriously compromised in spontaneously breathing preparations that received these drugs. Thus, the apnea which was observed in unanaesthetized spontaneously breathing mice following flufanemic acid and riluzole (Pena and Aguileta, 2007) might have been secondary to hypoventilation and not to a primary suppression of mechanisms of respiratory rhythm generation.

4.2 Limitations of findings from *in situ* preparation

Channels for calcium-activated non-specific cationic current and persistent sodium current are ubiquitous in the peripheral and central nervous system (see Ramirez and Viemari, 2005; Paton and St.-John, 2007). The marked reduction in amplitudes of phrenic and vagal discharges, but maintenance of rhythmic activities could reflect actions of blockers on neurons other than medullary bursters. Hence, burster activities might be continuing at concentrations of blockers which eliminated respiratory-modulated motoneuronal activities. Such a continuation of burster activities does not fit with findings following administrations of riluzole in which eupnea continues at concentrations several fold higher than those which resulted in an elimination of both the discharge of medullary burster neurons and of gasping (Paton *et al.*, 2006; St.-John *et al.*, 2007). For flufanemic acid, no such comparison of concentrations eliminating burster activities versus rhythmic respiratory-modulated activities of peripheral nerves is possible since this second type of burster neuronal activity has only been recorded, to date, in one specific type of medullary slice preparation of mouse (Pena *et al.*, 2004). Concerning this slice, the frequency of its rhythmic discharges declines significantly following administrations of flufanemic acid and declines further following riluzole, to a cessation of activity in some trials (Pena *et al.*, 2004). In the present study, the frequency of phrenic bursts increased following these drugs. While interpretation of such an increase is confounded by the finding of a time-dependent increase in the frequency of phrenic discharge of the *in situ* preparation, still some decline or the absence of an increase would have been expected if the discharge of medullary pacemakers was responsible for generating the eupneic rhythm.

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Figure 1.

Influence of flufanemic acid (FFA) on activities of the phrenic and vagus nerves. Upper panel (A) shows integrated activities of the phrenic (JPhr.) and vagus (JVag) nerves in eupnea. Middle panel (B) shows changes in these activities following addition of 30 μ M of FFA to the perfusate. Note increase in frequency of bursts, diminution of peak level in most cycles and marked reduction or loss of vagal expiratory discharge. Bottom panels (C) show recordings in gasping after FFA had been administered. Black bar shows period of exposure to hypoxic hypercapnia. Note decremting discharges of both nerves, which is typical of gasping.

Figure 2.

Alterations in activities of the phrenic and vagus nerves following additions to the perfusate of flufanemic acid and riluzole. Upper panel (A) shows integrated activities of the phrenic (JPhr.) and vagus (JVag) nerves in eupnea. Middle panel (B) shows changes in these activities following addition of 30 μ M of FFA and 10 μ M of riluzole to the perfusate. Note increase in frequency of bursts and diminution in peak level of integrated nerve activities. Bottom panel (C) show recordings in hypoxic hypercapnia. Black bar shows period of exposure to hypoxic hypercapnia. Note the cessation of discharge in hypoxia and the absence of any discharges typical of gasping.