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Temperature and pH/CO₂ modulate respiratory activity in the isolated brainstem of the bullfrog (*Rana catesbeiana*)

Rey D. Morales, Michael S. Hedrick*

Department of Biological Sciences, California State University, Hayward, Hayward, CA 94542, USA

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Abstract

The effects of temperature and pH/CO₂ were examined in isolated brainstem preparations from adult North American bullfrogs (*Rana catesbeiana*). These experiments were undertaken to determine the effects of temperature on fictive breathing, central pH/CO₂ chemoreception, and to examine potential alaphastat regulation of respiration in vitro. Adult bullfrog brainstem preparations were isolated, superfused with an artificial cerebrospinal fluid (aCSF) and respiratory-related neural activity was recorded from cranial nerves V, X and XII. In Series I experiments ($N=8$), brainstem preparations were superfused with aCSF equilibrated with 2% CO₂ at temperatures ranging from 10 to 30 °C. Neural activity was present in all preparations in the temperature range of 15–25 °C, but was absent in most preparations when aCSF was at 10 or 30 °C. The absence of fictive breathing at high (30 °C) temperatures was transient since fictive breathing could be restored upon returning the preparation to 20 °C. In Series II experiments ($N=10$), preparations were superfused with aCSF equilibrated with 0%, 2% and 5% CO₂ at temperatures of 15, 20 and 25 °C. Fictive breathing frequency (f_R) was significantly dependent upon aCSF pH at all three temperatures, with slopes ranging from $-0.82 \text{ min}^{-1} \text{ pH unit}^{-1}$ (15 °C) to $-3.3 \text{ min}^{-1} \text{ pH unit}^{-1}$ (20 °C). There was a significant difference in these slopes ($P < 0.02$), indicating that central chemoreceptor sensitivity increased over this temperature range. Fictive breathing frequency was significantly dependent upon the calculated alpha-imidazole (α_{Im}) ionization ($P < 0.05$), consistent with the alaphastat hypothesis for the effects of temperature on the regulation of ventilation. However, most of the variation in f_R was not explained by α_{Im} ($R^2=0.05$), suggesting that other factors account for the regulation of fictive breathing in this preparation. The results indicate that the in vitro brainstem preparation of adult bullfrogs has a limited temperature range (15–25 °C) over which fictive breathing is consistently active. Although there is a close correspondence of ventilation in vitro and in vivo at low temperatures, these data suggest that, as temperature increases, changes in ventilation in the intact animal are likely to be more dependent upon peripheral feedback which assumes a greater integrative role with respect to chemoreceptor drive, respiratory frequency and tidal volume. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Breathing in amphibians and other vertebrates is a behavioral motor activity that originates with a neural network localized to the medulla (Taylor et al., 1999; Wang et al., 1999). The neural

network, or central pattern generator (CPG), that provides motoneuronal output to respiratory muscles has not been clearly delineated in ectothermic vertebrates; however, in mammals, the pre-Bötzinger Complex is hypothesized to be the primary rhythm-generating region within the medulla (Smith et al., 1991; Gray et al., 2001). In recent years, examination of the respiratory CPG from a variety of vertebrates has made use of in vitro brainstem preparations that have rendered the prob-

*Corresponding author. Tel.: +1-510-885-7443; fax: +1-510-885-4747.

E-mail address: mhedrick@csuhayward.edu (M.S. Hedrick).

lem of studying this complex motor behavior slightly more tractable (Rovainen, 1983; Suzue, 1984; Feldman and Smith, 1989; Douse and Mitchell, 1990; McLean et al., 1995). There are a number of advantages to the use of in vitro preparations for examination of CPGs that have been previously enumerated (Mitchell, 1993; Luksch et al., 1996). However, there are some significant drawbacks of this preparation in endotherms, such as maintaining the preparation at a reduced temperature to maintain viability and measurements by some workers indicating that the core of the preparation is anoxic and highly acidic (Okada et al., 1993). In contrast, brainstem preparations from ectothermic vertebrates such as lamprey (Rovainen, 1983), larval and adult amphibians (Kinkead et al., 1994; McLean et al., 1995; Torgerson et al., 1997a; Hedrick et al., 1998; Reid and Milsom, 1998; Broch et al., 2002) and reptiles (Douse and Mitchell, 1990; Johnson et al., 1998) can be maintained at the normal temperatures experienced by the animals. Measurements of PO_2 and pH within the core of the tadpole brainstem in vitro indicate that the preparation is normoxic or slightly hyperoxic (Torgerson et al., 1997b), and it is likely that brainstem–spinal cord preparations from other ectotherms exhibit similar PO_2 and pH profiles. Moreover, with the lower tissue metabolism and greater tissue oxygenation, preparations from ectotherms are viable for much longer periods of time (several hours or days) compared with mammalian preparations.

Several recent studies have used larval or adult amphibian brainstem–spinal cord preparations to examine the control of the respiratory CPG in vertebrates. There is evidence that central chemosensitivity is retained in this preparation because fictive breathing has been shown to increase with changes in the level of pH/ CO_2 in the perfusion fluid (Kinkead et al., 1994; McLean et al., 1995; Torgerson et al., 1997a). However, each of these studies has examined the effects of pH/ CO_2 at a temperature of approximately 20 °C. Because most amphibians experience daily or seasonal changes in body temperature, it is important to determine the extent to which the respiratory CPG is also influenced by the range of temperatures normally experienced by these animals (Hutchison and Dupre, 1992; Ultsch and Jackson, 1996).

The precise role of temperature and its effects on pH, central chemoreception and breathing in ectotherms are somewhat controversial. Reeves

(1972) hypothesized that the key regulated variable with respect to ventilation in ectotherms is the fractional dissociation of alpha-imidazole (α_{im}) groups of the amino acid histidine. This ‘alphastat’ hypothesis explains inverse relationship between temperature and blood pH in a large number of ectotherms (see Ultsch and Jackson, 1996). However, it has been difficult to evaluate this hypothesis in vivo owing to the interaction of central and peripheral chemoreceptors and their dependence upon O_2 and/or CO_2 . Because there are no studies that have examined the effects of changing temperature and pH/ CO_2 on respiratory-related activity from amphibians in vitro, the present study affords an opportunity to examine the alphastat hypothesis using an isolated, fictively-breathing bullfrog brainstem preparation. The specific aims of this study were to determine the temperature range over which the adult bullfrog brainstem preparation in vitro is able to maintain respiratory-related neural output, and to what extent temperature influences central chemosensitivity to pH/ CO_2 and fictive ventilation, in the context of the alphastat hypothesis.

2. Material and methods

2.1. Animals

North American bullfrogs (*Rana catesbeiana*: 110–160 g; $N=18$) were used in this study and purchased from a commercial supplier (West Jersey Bio Services, Inc., Wenonah, NJ, USA). The frogs were kept in fiberglass tanks supplied with fresh running tap-water at room temperature (20–23 °C).

2.2. In vitro brainstem preparation

For experiments, frogs were anesthetized in a buffered solution of tricaine methanesulfonate (MS-222; pH 7.8), decerebrated under anesthesia, and the brain removed as previously described (Hedrick et al., 1998). The brainstem was placed into a recording chamber (7 ml) and superfused with artificial cerebrospinal fluid (aCSF) with the following composition (in mM): 75 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 1.0 $NaHPO_4$, 40 $NaHCO_3$ and 7.5 glucose (Kinkead et al., 1994). The aCSF flowed through the recording chamber at 5–10 ml/min from a gravity-fed reservoir (350 ml) initially maintained to achieve a temperature

of 10 °C (series I) or 15 °C (series II) (see below). The aCSF reservoir was initially bubbled with oxygenated, isocapnic (98% O₂/2% CO₂) gas from an electronic mixing flowmeter (Cameron Instrument Co., model GF-3MP). Neural output was recorded with suction electrodes attached to cranial motoneurons V (trigeminal), X (vagus) and XII (hypoglossal). These nerves innervate buccal elevator and depressor muscles in the oropharyngeal region of anurans and are responsible for generating airflow, and controlling glottal airflow, associated with small amplitude, non-ventilatory buccal oscillations and larger amplitude, positive-pressure lung ventilatory events (DeJongh and Gans, 1969; Kinkead and Milsom, 1994; Kogo et al., 1994). Previous studies with adult amphibian preparations have verified that neural activity is correlated with breathing movements in intact animals (Sakakibara, 1984). Neural activity was amplified (A-M Systems, model 1700), filtered and stored on computer (Power Macintosh 7200) that interfaced with a data acquisition system sampling at 2 kHz (AD Instruments; MacLab/8S).

The aCSF temperature was controlled with a constant temperature water bath (Lauda, model RM6) that circulated water through the outside jacket of the reservoir. Temperature of the aCSF within the recording chamber was monitored continuously with a thermocouple (Cole-Parmer, model 8402-00) placed approximately 0.5 cm from the brainstem. The aCSF pH and temperature were measured continuously in the reservoir with a digital pH meter (Jenco Electronics) and mercury thermometer to within 0.5 °C. In preliminary experiments, the relationship between pH and temperature in the reservoir was established for a broad range of temperatures (5–30 °C) and CO₂ levels (0%, 2% and 5% CO₂, bal. O₂). There were highly significant, linear relationships ($R^2 > 0.95$) between pH and temperature at all three levels of CO₂. These linear relationships were used to calculate pH in the recording chamber at any particular temperature and CO₂ level. In a separate set of experiments, the possibility of CO₂ diffusion between the reservoir and recording chamber was examined by measuring pH simultaneously in the reservoir and in the recording chamber with no brainstem preparation present. There was no significant difference between reservoir pH and recording chamber pH when measured at equivalent temperatures, indicating that no significant

diffusion of CO₂ occurred between the reservoir and the recording chamber.

2.3. Experimental protocol

2.3.1. Series I

In a first set of experiments ($N=8$), we determined the range of temperatures at which neural activity was present when the brainstem was equilibrated with the control aCSF (98% O₂/2% CO₂). In these experiments, after the brainstem was transferred to the recording chamber, temperature was held at 10 °C for 30–60 min and any neural activity recorded. Temperature was raised in 5 °C increments every 30 min until a final temperature of 30 °C was reached. Each step change in temperature required approximately 5 min to complete. Neural activity was recorded at each temperature interval for 5 min before raising the temperature of the recording chamber.

2.3.2. Series II

Following the series I experiments, a second set of experiments (Series II; $N=10$) was done to determine the effects of a more limited temperature range (15–25 °C) on neural activity at different levels of pH/CO₂. After the brainstem was transferred to the recording chamber, and before any measurements were taken, the brainstem was superfused continuously with aCSF equilibrated with 98% O₂/2% CO₂ for 1 h at 15 ± 0.2 °C (S.E.M.). The 2% CO₂ perfusate was considered the 'control' aCSF because this approximates the CO₂ level of arterial blood in adult bullfrogs (Kinkead and Milsom, 1994). After a 1-h equilibration period, a 5-min sample of neural activity was recorded. Following this, the aCSF was bubbled with 100% O₂ (0% CO₂) and maintained for 30 min. Neural activity was recorded during the 25–30-min period of equilibration. The aCSF was switched to 95% O₂/5% CO₂ for 30 min and a recording taken during the 25–30-min period of equilibration. After all three levels of CO₂ were used (2%, 0% and 5% CO₂), the temperature was increased until the temperature in the recording chamber was 20 ± 0.1 °C and held at this temperature for 30 min. The CO₂ equilibration protocol was repeated, with a 5-min sample of neural activity recorded during the 25–30-min equilibration period with each level of CO₂. The temperature was then raised to 25 ± 0.2 °C, and a 5-min

recording taken at each 30 min level of CO₂ equilibration.

2.4. Data analysis and statistics

In some preparations, neural activity was present as high frequency, low amplitude bursts associated with buccal oscillations; however, all preparations exhibited low frequency, high amplitude bursts associated with lung ventilation (Kinkead et al., 1994; McLean et al., 1995; Hedrick et al., 1998). Neural activity was considered a lung burst if the integrated burst amplitude was at least twice as large as the average integrated buccal amplitude.

A two-way analysis of variance (ANOVA) was used to test for significant effects of temperature and CO₂ on fictive breathing. Linear regression analysis was done to examine the effects of aCSF pH on fictive breathing. Differences between regression slopes and intercepts were done with Analysis of Covariance (ANCOVA) as outlined in Zar (1974). Measured respiratory variables included respiratory frequency (f_R), burst duration and relative burst amplitude. Neural minute activity was calculated as $f_R \times$ amplitude. Significant differences detected by ANOVA were analyzed post hoc by the Student–Newman–Keuls multiple range test (Zar, 1974). All statistical tests were done using commercially available statistics/graphics programs (GraphPad Prism, v. 3.0, GraphPad Software, Inc., and Igor v. 4.0.1, WaveMetrics, Inc.).

3. Results

3.1. Series I experiments

Series I experiments examined the temperature range over which the adult bullfrog brainstem preparation exhibited activity. For Series I experiments, aCSF pH ranged from approximately 7.37 (10 °C) to 7.67 (30 °C). Each preparation exhibited respiratory-related neural activity at 15, 20 and 25 °C (Table 1). However, at the lowest temperature used (10 °C) neural activity was present in one of eight preparations, and only two of eight preparations exhibited any activity at 30 °C. Fig. 1 illustrates the effects of temperature changes in a single preparation. In this experiment, high frequency, low amplitude buccal activity was present in addition to high amplitude, low frequency lung bursts. Overall, fictive breathing frequency

Table 1

Number of preparations exhibiting respiratory-related neural activity and fictive lung burst frequency for Series I brainstem preparations ($N=8$ total; see text)

	Temperature (°C)				
	10	15	20	25	30
Number of preparations with activity	1	8	8	8	2
Frequency ^a (bursts min ⁻¹)	0.2	2.1 ± 0.5	2.6 ± 0.7	2.2 ± 0.8	2.8, 1.6

^a Values are mean ± S.E.M. for $N > 2$.

(f_R) ranged from 2.1 ± 0.5 breaths min⁻¹ at 15 °C to 2.6 ± 0.7 breaths min⁻¹ at 20 °C (Table 1), but there was no significant difference in f_R over this temperature range (ANOVA; $F_{2,23}=0.18$; $P > 0.5$).

In two experiments, we examined whether the cessation of respiratory activity at 30 °C was a transient effect, or if the preparation was incapable of producing activity after exposure to high temperature. In both experiments, respiratory-related neural activity resumed, albeit after a delay, following exposure to high (30 °C) temperature, and subsequently lowering temperature to 20 °C. In the experiment shown in Fig. 2, neural activity began at 15 °C and aCSF temperature was increased to 20 °C (Fig. 2a) and then to 25 °C (Fig. 2b). Neural activity ceased when temperature was raised to 30 °C (data not shown, but see Fig. 1), but resumed again when temperature was returned to 20 °C a second time (Fig. 2c). Neural activity in this preparation was absent for approximately 2 h during and immediately following exposure to 30 °C, but returned to a similar frequency following the exposure to high temperature. Aside from an increase in burst amplitude, there appeared to be no fundamental difference in neural burst characteristics before and after exposure to 30 °C.

3.2. Series II experiments

Based upon our findings from series I experiments, we examined the effects of changes in aCSF CO₂ over a limited temperature range (15–25 °C). Fig. 3 illustrates the effect of different concentrations of CO₂ on fictive ventilation in one experiment at 15, 20 and 25 °C. There was a significant effect of pH on f_R at all three temper-

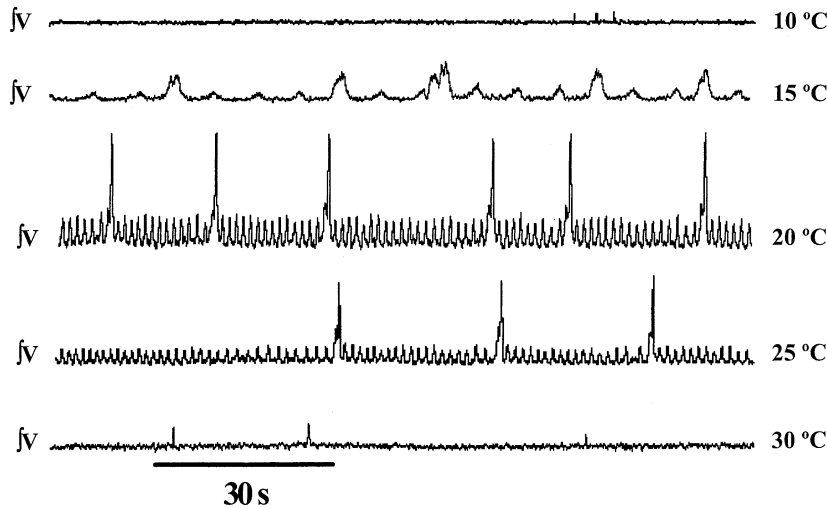


Fig. 1. Integrated neural activity from trigeminal nerve $\left(\int V\right)$ for a single experiment (Series I). Artificial CSF was bubbled with 2% CO_2 and temperature was increased from 10 to 30 °C in 5 °C increments. See text for details.

atures (Fig. 4a). The regression equations describing the dependence of aCSF pH on f_R were: 15 °C, $f_R = 7.14 - 0.82(\pm 0.26) \times \text{pH}$ ($R^2 = 0.26$; $F_{1,27} = 9.6$; $P < 0.005$); 20 °C, $f_R = 28.5 - 3.3(\pm 0.74) \times \text{pH}$ ($R^2 = 0.41$; $F_{1,28} = 19.9$; $P < 0.001$); 25 °C, $f_R = 14.1 - 1.52(\pm 0.65) \times \text{pH}$ ($R^2 = 0.20$; $F_{1,22} = 5.4$; $P < 0.03$). Similar relationships

between neural minute activity ($f_R \times \text{amplitude}$) and pH were measured (Fig. 4b) because there was no significant effect of temperature or pH on lung burst amplitude (Fig. 5c). There was a significant difference among the slopes of these regressions (ANCOVA; $F_{2,77} = 4.8$; $P < 0.02$), sug-

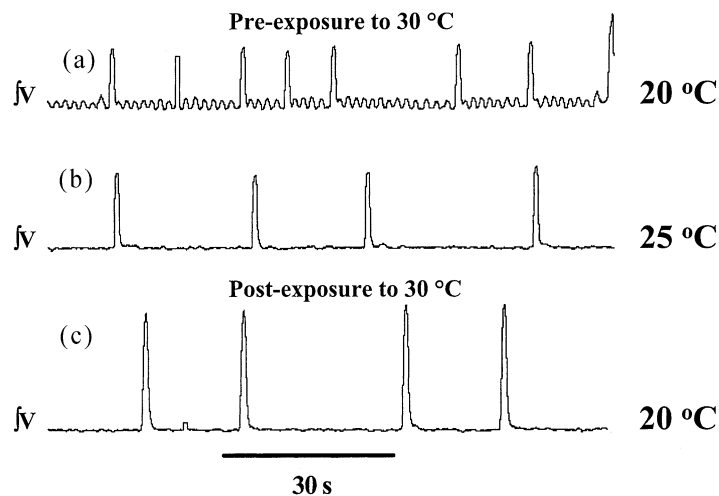


Fig. 2. Effects of high temperature (30 °C) on a single Series I preparation. Integrated neural activity $\left(\int V\right)$ is shown under control conditions (a, 20 °C, 2% CO_2). Temperature of aCSF was increased to 25 °C (b) and then to 30 °C (data not shown; see Fig. 1) for 30 min with no neural activity observed. Temperature was lowered to 20 °C and neural activity resumed after a delay of 75 min (c) with an increased amplitude.

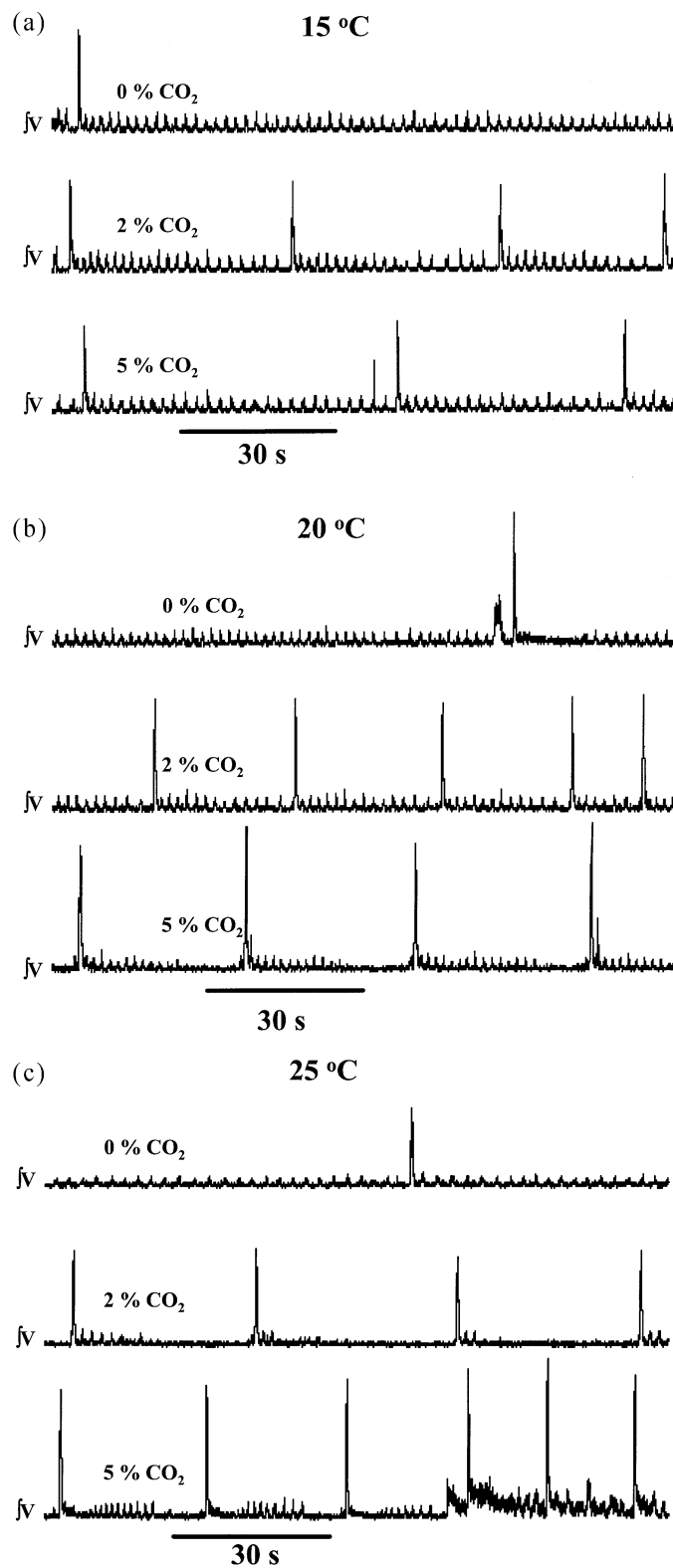


Fig. 3. Integrated trigeminal activity $\left(\int V\right)$ for a single experiment (Series II) at three levels of aCSF CO₂ (0%, 2%, 5% CO₂) at 15 °C (a), 20 °C (b) and 25 °C (c). See text for details.

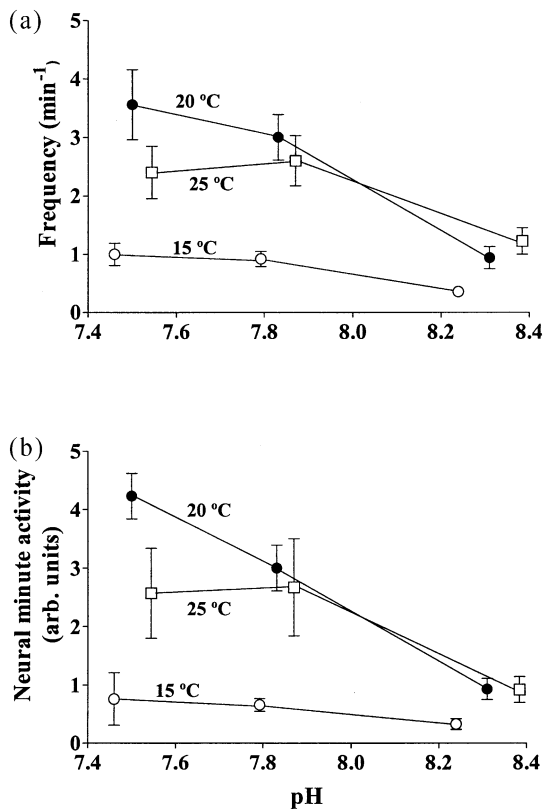


Fig. 4. Effect of aCSF pH on lung burst frequency (a) and neural minute activity (b) at three temperatures (unfilled circles, 15 °C; filled circles, 20 °C; squares, 25 °C) for Series II experiments. Values are mean \pm S.E. ($N=10$).

gesting that central chemoreceptor ‘sensitivity’ changes as a function of temperature.

Fig. 5 summarizes the effects of temperature on respiratory activity at the three levels of aCSF CO_2 . There was a significant increase in f_R and neural minute activity when temperature was increased from 15 to 20 °C at 2% and 5% CO_2 , but not at 0% CO_2 (Fig. 5a,b). There was no further change in fictive breathing when temperature was raised to 25 °C at any CO_2 level. Thus, fictive breathing was maximal at a temperature range of 20–25 °C, with no difference between equilibration with 2% CO_2 or 5% CO_2 (Fig. 5a,b). The increase in f_R over the 15–20 °C range correspond to Q_{10} values ranging from 6.7 to 9.5, but when measured over the 15–25 °C temperature range, Q_{10} is 1.0–1.9. There was no significant effect of aCSF temperature or aCSF CO_2 on fictive burst amplitude or burst duration (Fig. 5c,d).

Fictive breathing frequency expressed as a function of the calculated α_{Im} for all data in Series II experiments are shown in Fig. 6a. The calculated α_{Im} ranged from approximately 0.66 to 0.96 and was determined using equations described by Reeves (1972, 1976). There was a significant relationship between f_R and α_{Im} ($f_R = 4.8 - 3.6(\pm 1.7) \times \alpha_{\text{Im}}$; $F_{1,83} = 4.43$; $P < 0.05$). However, only 5% of the variation in f_R could be explained by α_{Im} ($R^2 = 0.05$). Fig. 6b shows summarized f_R as a function α_{Im} at the three temperatures (15, 20 and 25 °C) used in the series II experiments.

4. Discussion

Several previous studies have examined the effects of pH, or the role of neurotransmitters, on fictive breathing in larval or adult bullfrogs at a temperature of approximately 20 °C (Kinkead et al., 1994; McLean et al., 1995; Torgerson et al., 1997a; Reid and Milsom, 1998; Hedrick et al., 1998; Broch et al., 2002). In general, these studies indicate that the in vitro bullfrog preparation responds to changes in pH/ CO_2 of the perfusion fluid surrounding the brainstem. The present study confirms previous results at 20 °C and extends these results by examining fictive breathing in this preparation over a wider range of temperatures (10–30 °C) that would normally be experienced by bullfrogs in temperate regions (Hutchison and Dupre, 1992; Ultsch and Jackson, 1996).

4.1. Effects of temperature on fictive ventilation

Fictive ventilation was present in all Series I preparations between 15 and 25 °C, but was nearly absent at low (10 °C) or high (30 °C) temperatures (Table 1). At 10 °C, our failure to detect fictive breathing in most preparations may be due, in some part, to a sampling error since we recorded activity for 5 min, which may have been too short a time to detect low breathing frequencies (< 0.2 breaths min^{-1}). A more likely explanation is that low temperatures interfere with synaptic transmission to respiratory motoneurons and/or cause ventilatory arrest at the level of the central rhythm-generating network. Because we determined respiratory-related neural activity by measuring output from cranial motoneurons, our results do not allow us to distinguish between these different possible mechanisms. Low temperatures are known to decrease mean glutamate channel

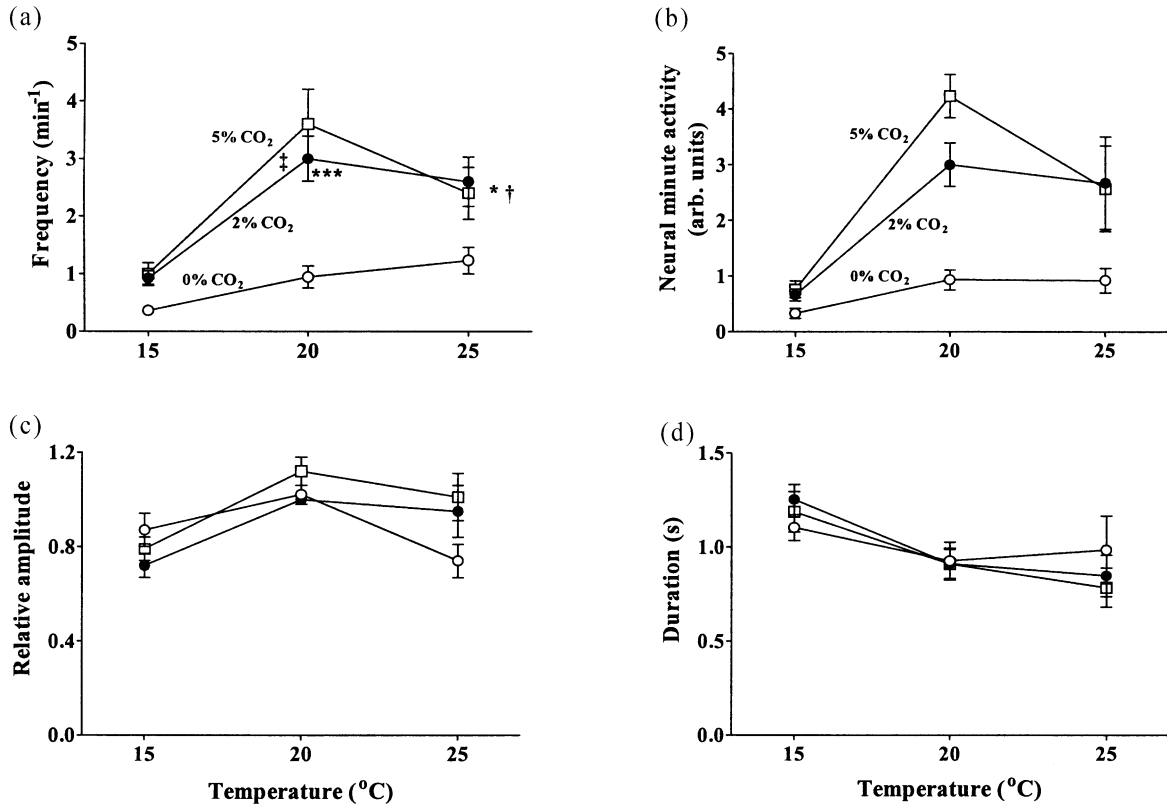


Fig. 5. Effect of aCSF temperature on lung burst frequency (a), neural minute activity (b), relative burst amplitude (c) and lung burst duration (d) at three levels of aCSF CO₂ (unfilled circles, 0% CO₂; filled circles, 2% CO₂; squares, 5% CO₂). Values are mean ± S.E. (N=10). ***P < 0.001, *P < 0.05 compared with 15 °C (2% CO₂ and 5% CO₂); †P < 0.001 compared with 20 °C (0% CO₂); ‡P < 0.05 compared with 25 °C (0% CO₂).

conductance (Anderson et al., 1977). Because glutamate is an important excitatory neurotransmitter in the amphibian brainstem (McLean et al., 1995), a reduction in glutamate channel activity at low temperatures might have important effects on synaptic transmission and fictive breathing in this preparation. A recent study using a neonatal rat brainstem slice preparation suggests that cooling arrests fictive breathing at the level of the rhythm-generating network (Mellen et al., 2002).

When temperature was raised to 30 °C, fictive breathing ceased in most preparations (Table 1). The cessation of neural activity apparently was not due to death of CPG neurons because neural activity could be restored upon lowering temperature to 20 °C following exposure to 30 °C (Fig. 2). The reasons for the cessation of activity at 30 °C are unclear, but could involve a number of possible mechanisms. For example, increased temperature may lead to oxygen limitations and failure to maintain adequate energy production in this

preparation. Oxygen diffusion limitation is a problem in the whole isolated mammalian brainstem preparation (see Feldman and Smith, 1989; Okada et al., 1993), and this has led to the development of brainstem slice preparations that maintain respiratory-related neural activity. However, the bullfrog tadpole brainstem preparation is well-oxygenated ($P_{O_2} \approx 150$ torr) and relatively non-acidic within the core of the brain tissue at 22 °C (Torgerson et al., 1997b). Although reductions in tissue P_{O_2} , or increased acidity, might account for a cessation of fictive breathing in our experiments, it seems unlikely that tissue P_{O_2} would fall to levels that would compromise energy production when tissue temperature increased to 30 °C. The brain of *Rana pipiens* can survive complete anoxia for 1–2 h at 25 °C despite a reduction of brain [ATP] to 20% of normoxic values (Lutz and Reiners, 1997). Another possible mechanism is that temperature-dependent ion channels, such as the tandem-pore (2P) K⁺ channels, are activated

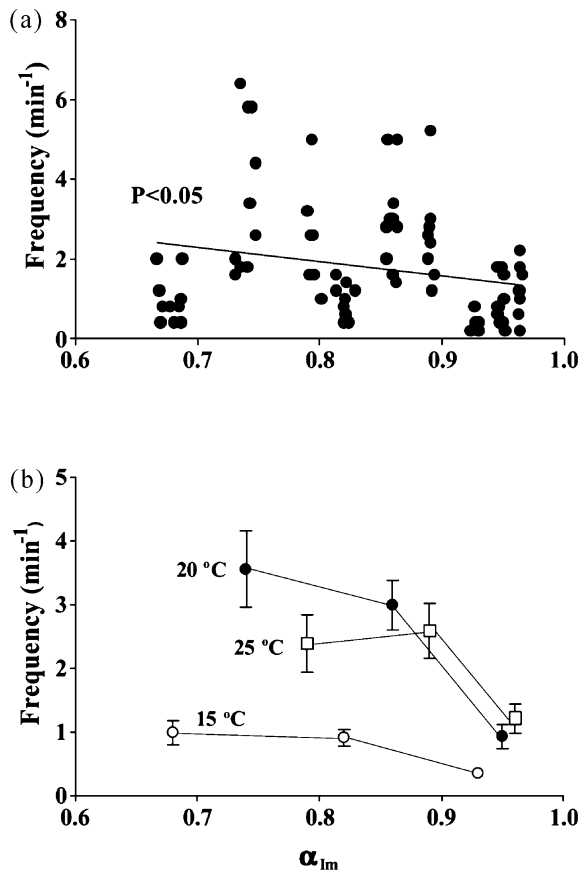


Fig. 6. Fictive lung burst frequency (min^{-1}) as a function of the calculated alpha-imidazole (α_{im}) for series II experiments. (a) For all data, there was a significant effect of α_{im} on lung burst frequency ($P < 0.05$; $F_{1,81} = 4.43$; $R^2 = 0.05$). (b) Summary of effects of α_{im} on fictive breathing frequency for preparations at 15 °C (unfilled circles), 20 °C (filled circles) and 25 °C (squares).

at higher temperatures (Patel and Honore, 2001). A subset of this class of K^+ channel—the TREK-1 channel—is reversibly opened by increased temperature (Maingret et al., 2000) and this would have the effect of shunting current, thus making depolarization more difficult in regions where this channel is expressed.

4.2. Effects of temperature and pH/ CO_2 on fictive breathing

Based upon our results from the Series I experiments, we examined the effects of temperature changes in the range over which this preparation is active (15–25 °C) and examined the effects of pH/ CO_2 at these temperatures. There is a close

correspondence between fictive breathing frequency and breathing rates in conscious bullfrogs in the lower temperature range. In the present study, and in other studies with the adult bullfrog brainstem preparation, fictive breathing rates are in the range of approximately 1–10 breaths min^{-1} depending on the pH of the perfusion fluid (Kinkead et al., 1994; McLean et al., 1995; Reid and Milsom, 1998; Hedrick et al., 1998; Broch et al., 2002). Fictive breathing frequency in the present study was approximately 1–2 breaths min^{-1} at 10–15 °C, which is nearly identical to rates recorded for intact bullfrogs at 10 °C (Rocha and Branco, 1998). At temperatures of 20 °C and above, however, there are marked differences in respiratory activity and the response to temperature changes between the in vitro preparation and the intact animal. In conscious bullfrogs and toads (*Bufo paracnemis*), breathing frequency is approximately 5–20 breaths min^{-1} at 20–25 °C, depending on the season in which the measurements are taken (Rocha and Branco, 1998).

Temperature also has a significant effect on hypercapnia-induced stimulation of breathing in the intact animal (Bicego-Nahas and Branco, 1999); however, this response is blunted in the amphibian brainstem preparation (this study; Kinkead et al., 1994; McLean et al., 1995; Reid and Milsom, 1998) and reptiles (Johnson et al., 1998). This blunted central chemoreceptor response in vitro can be largely accounted for by the elimination of sensory, primarily vagal, feedback. Vagotomy in the intact, or decerebrate, artificially-ventilated, bullfrog significantly reduces responses to hypercapnia (Milsom et al., 1999) and stimulation of vagal afferents in the in vitro adult bullfrog preparation significantly enhances fictive breathing and central chemoreceptor sensitivity to aCSF pH (Kinkead et al., 1994). A similar study using a turtle (*Chrysemys picta*) in vitro brainstem preparation examined the effects of temperature and pH/ CO_2 on fictive breathing (Johnson et al., 1998). Their study found a significant effect of pH/ CO_2 on fictive breathing, but that increasing aCSF temperature from 22 to 32 °C did not increase central chemoreceptor sensitivity, in contrast to data from intact turtles. The Q_{10} for peripheral (vagal) feedback is 3.0 or more in reptiles (Douse et al., 1989) and perhaps in other ectotherms. This suggests that as temperature increases, peripheral feedback from chemoreceptors, lung stretch receptors, and other afferent

inputs to the CNS that regulate respiratory activity, would assume a greater degree of importance in determining the final motor output from the brainstem central pattern generator.

The bullfrog brainstem preparation exhibited an increase in central chemoreceptor sensitivity that was maximal ($-3.3 \text{ min}^{-1} \text{ pH unit}^{-1}$) at 20°C and declined slightly at 25°C (Fig. 4). Since this temperature range is near the preferred body temperature of North American bullfrogs (Hutchison and Dupre, 1992), a maximal central chemoreceptor sensitivity may be important for enhancing respiratory responses and integrating peripheral feedback at these temperatures. It should be emphasized, however, that the in vitro changes in central chemosensitivity are small compared with temperature and CO_2 -induced changes in breathing in the intact animal.

4.3. Alaphastat regulation of breathing

There is a well-characterized inverse relationship between body temperature and blood pH for ectothermic vertebrates. In order to explain this relationship, Reeves (1972, 1976) proposed that ectotherms regulate the fractional dissociation state of alpha-imidazole (α_{Im}) groups of histidine. The term 'alaphastat' is used to characterize the importance of this particular protein buffer system. The alaphastat hypothesis has been difficult to test directly, although there is considerable indirect evidence for its validity (see Ultsch and Jackson, 1996). A key feature of this 'alaphastat hypothesis' is that ventilation is regulated in order to maintain a constant α_{Im} when temperature changes. This hypothesis also predicts that ventilation should vary directly with changes in α_{Im} regardless of temperature (Hitzig, 1982). Studies in turtles and goats in which central chemoreceptors were stimulated by ventricular perfusion independently of peripheral inputs showed that ventilation was significantly dependent upon α_{Im} , thus providing strong evidence for the alaphastat hypothesis in vivo (Hitzig, 1982). In addition, ventilatory activity is blocked with an imidazole-binding agent, diethylpyrocarbonate, in the in vitro rat brainstem preparation, suggesting that functional integrity of imidazole groups plays an important role in maintaining ventilatory activity (Krause et al., 1998).

The alaphastat hypothesis has been somewhat controversial and testing this hypothesis is complicated by the interaction of central and peripheral

chemoreceptors, mechanoreceptors and their effects on ventilation (see Milsom et al., 1999). The present study, using an in vitro brainstem preparation from an ectotherm, affords an opportunity to examine the alaphastat hypothesis without the potential complication of peripheral feedback. Our results indicate that fictive breathing is significantly dependent upon α_{Im} (Fig. 6a), however, there is a large degree of variability in this relationship in vitro, since only 5% of the variation in fictive breathing could be explained by α_{Im} . In this respect, our results are very similar to those of Branco et al. (1993) using toads with central perfusion of artificial CSF at different body temperatures. Their data, and results from this study (Fig. 6b), indicate that alaphastat regulation cannot fully account for the effects of temperature on breathing in amphibians. Taken together, there appears to be a complex interaction of peripheral input with central chemoreceptor activity to produce ventilatory motor output in amphibians. More studies will have to be done to fully understand the mechanisms underlying these interactions.

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