

Developmental Changes in the Modulation of Respiratory Rhythm Generation by Extracellular K^+ in the Isolated Bullfrog Brainstem

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ABSTRACT: This study tested the hypothesis that voltage-dependent, respiratory-related activity *in vitro*, inferred from changes in $[K^+]_o$, changes during development in the amphibian brainstem. Respiratory-related neural activity was recorded from cranial nerve roots in isolated brainstem–spinal cord preparations from 7 premetamorphic tadpoles and 10 adults. Changes in fictive gill/lung activity in tadpoles and buccal/lung activity in adults were examined during superfusion with artificial CSF (aCSF) with $[K^+]_o$ ranging from 1 to 12 mM (4 mM control). In tadpoles, both fictive gill burst frequency (f_{gill}) and lung burst frequency (f_{lung}) were significantly dependent upon $[K^+]_o$ ($r^2 > 0.75$; $p < 0.001$) from 1 to 10 mM K^+ , and there was a strong correlation between f_{gill} and f_{lung} ($r^2 = 0.65$; $p < 0.001$). When $[K^+]_o$ was raised to 12 mM, there was a reversible abolition of fictive breathing. In adults, fictive buccal frequency (f_{buccal}), was significantly dependent on $[K^+]_o$ ($r^2 = 0.47$; $p < 0.001$), but $[K^+]_o$

had no effect on f_{lung} ($p > 0.2$), and there was no significant correlation between f_{buccal} and f_{lung} . These data suggest that the neural networks driving gill and lung burst activity in tadpoles may be strongly voltage modulated. In adults, buccal activity, the proposed remnant of gill ventilation in adults, also appears to be voltage dependent, but is not correlated with lung burst activity. These results suggest that lung burst activity in amphibians may shift from a “voltage-dependent” state to a “voltage-independent” state during development. This is consistent with the hypothesis that the fundamental mechanisms generating respiratory rhythm in the amphibian brainstem change during development. We hypothesize that lung respiratory rhythm generation in amphibians undergoes a developmental change from a pacemaker to network-driven process. © 2003 Wiley Periodicals, Inc. *J Neurobiol* 55: 278–287, 2003

Keywords: respiratory rhythm generation; amphibian; pacemaker; neural network; potassium; development

INTRODUCTION

Respiratory rhythm in vertebrates is generated by a neuronal network localized to the medulla. There is considerable debate as to whether respiratory rhythm is generated primarily by a population of neurons with conditional pacemaker-like properties, by inhibitory

synaptic network interactions or by some combination of the two (Smith et al., 1991; Richter et al., 1992; Rekling and Feldman, 1998; Smith et al., 2000). Pacemaker cells with intrinsic, voltage-dependent bursting properties have been identified within the pre-Bötzinger complex (PBC) of neonatal rats and mice *in vitro* (Smith et al., 1991; Johnson et al., 1994; Rekling and Feldman, 1998; Koshiya and Smith, 1999; Thoby-Brisson and Ramirez, 2001). Although the PBC appears to be an important brainstem region for normal breathing in anesthetized cats (Pierrefiche et al., 1998; Ramirez et al., 1998) and awake rats (Gray et al., 2001), the contribution of pacemaker cells to respiratory rhythm generation has been difficult to

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verify experimentally (Del Negro et al., 2002). Respiratory rhythm generation is also hypothesized to be generated by neural networks that use synaptic inhibition (Richter et al., 1992), but do not require endogenously bursting pacemaker neurons. Recent models have incorporated elements of pacemaker and network models in which pacemaker neurons are imbedded in a neural network consisting of excitatory and inhibitory connections (Koshiya and Smith, 1999; Butera et al., 1999a, 1999b; Smith et al., 2000; Del Negro et al., 2001).

A recent version of the general network model, a “switching” hypothesis (Rybak et al., 2002; St-John et al., 2002), suggests that increases in extracellular potassium concentration ($[K^+]_o$) allows the expression of voltage-dependent bursting activity in PBC pacemaker cells (Koshiya and Smith, 1999; Del Negro et al., 2001; Johnson et al., 2001), that are suppressed at normal $[K^+]_o$. At present, there is supporting evidence for both network and pacemaker hypotheses, and it is difficult to evaluate which elements of either model are important for respiratory rhythm generation. Some of the difficulty in evaluating the pacemaker and network models in mammals is that a variety of experimental preparations, from *in vitro* slices from neonatal animals to awake behaving adult animals, have been used to test the various models (see Richter and Spyer, 2001, for review). Thus, both the type of preparation used and the stage of development contribute to the difficulty in evaluating models of respiratory rhythm generation.

Recent experiments using nonmammalian brainstem preparations have produced some insights into the development of respiratory rhythm generation. Nonmammalian models have some advantages over mammalian models, including the ability to use a single model (isolated brainstem-spinal cord) at all stages of development (see Luksch et al., 1996; Broch et al., 2002). For example, superfusion of larval and adult isolated bullfrog brainstem preparations with chloride-free artificial cerebrospinal fluid (aCSF), and antagonists of glycine and GABA_A receptors, indicates that lung burst generation may undergo a developmental switch from a pacemaker-driven to a network-driven process (Broch et al., 2002), as hypothesized in mammals (Richter and Spyer, 2001). Recent experiments with adult turtle brainstem-spinal cord indicate that respiratory rhythm also may be driven in a pacemaker-like manner (Johnson et al., 2002). The contribution of increased “excitability,” or voltage-dependent modulation, to respiratory rhythm generation in nonmammalian preparations is unknown.

In the present study, we have examined the effects

of changes in $[K^+]_o$ on respiratory-related motor output in the *in vitro* brainstem-spinal cord preparation of larval and adult bullfrogs. Because several models of respiratory rhythmogenesis rely upon voltage-dependent processes (Butera et al., 1999a, 1999b; Smith et al., 2000; Del Negro et al., 2001; Rybak et al., 2002), we have used changes in $[K^+]_o$ to infer voltage-dependent modulation of respiratory-related neural activity in brainstem preparations from larval and adult amphibians. Some of these data have appeared previously in abstract form (Wade and Hedrick, 2002).

METHODS

Animals

Experiments were performed on 10 adult (body mass 34 to 306 g) and 7 larval (premetamorphic tadpoles) (Taylor-Köllros, 1946) (T-K) stages V–XII; body mass 3.9 to 14.3 g) North American Bullfrogs (*Rana catesbeiana*). Animals were purchased from a commercial supplier (Charles D. Sullivan Co., Inc.; Nashville, TN). Adults were maintained in plastic tanks with continuous access to water; tadpoles were kept in fiberglass aquaria with aerated, dechlorinated tapwater. All animals were maintained at room temperature (20–23°C). All experimental procedures were approved by the CSUH Institutional Animal Care and Use Committee.

In Vitro Brainstem Preparation

Prior to surgery, animals were anesthetized by submersion in an aqueous solution of ethyl-*m*-aminobenzoate (MS-222, Sigma Chemical Co., St. Louis, MO; adults: 1.5 g/L; tadpoles: 0.5 g/L) buffered to pH 7.8 with sodium bicarbonate. When breathing movements ceased and the withdrawal and corneal reflexes were abolished (adults: 10–20 min; tadpoles: 2–5 min), animals were removed from the MS-222 solution and placed in ice water for 1 h to slow metabolism and maintain anesthesia for subsequent dissection.

A small opening was then made in the cranium with a dental drill, allowing for the transection and removal of the forebrain rostral to the optic lobes. During decerebration and subsequent dissection, the brainstem was constantly perfused with cold (5–10°C) artificial CSF (aCSF) with the following composition (mM): adult—NaCl, 75.0; KCl, 4.0; MgCl₂, 1.0; NaH₂PO₄, 1.0; NaHCO₃, 40.0; CaCl₂, 2.5; glucose, 5.0; tadpole —NaCl, 104.0; KCl, 4.0; MgCO₂, 1.4; NaHCO₃, 25.0, CaCl₂, 2.4; glucose, 10.0: (Kinkead et al., 1994; Torgerson et al., 2001), and equilibrated with 98% O₂/2% CO₂. The spinal cord was transected caudal to the brachial nerves and cranial nerve roots were severed at their exit from the cranium. The entire dissection required approximately 30 min to complete.

The isolated brainstem was pinned ventral side up in a

syngard-lined (Dow Corning) recording chamber (7 mL) and the dura and arachnoid were removed. Throughout this process, and during all subsequent experiments, the recording chamber was continuously perfused with oxygenated aCSF (pH 7.8, 20°C) from a gravity-fed reservoir (350 mL) at a flow rate of 5–10 mL/min (Morales and Hedrick, 2002).

Suction electrodes, fabricated from thin-walled capillary glass and held in micromanipulators (Narashige), were attached to the nerve roots of cranial nerves (CN) V (trigeminal), X (vagus), and XII (hypoglossal) in the adult preparation and CN V, VII (facial), and XII in the tadpole. Nerve activity was amplified 10,000 times with a differential AC amplifier (A-M systems model 1700; Everett, WA), filtered (100 Hz–5 kHz) and recorded on a computer that interfaced with a data acquisition system sampling at 2 kHz (Powerlab 8/SP; Chart v. 4.0; AD Instruments, Milford, MA).

Experimental Protocol

The brainstem preparation was superfused with aCSF for 1 h, or until the signal was stable, before a 10-min control recording was obtained. An initial control recording ($[K^+]_o = 4 \text{ mM}$) was followed by 15-min superfusions of aCSF with varying $[K^+]_o$ (1–12 mM). For each experiment, superfusion with aCSF began and ended with the control superfusate (4 mM $[K^+]_o$). Respiratory-related motor output from the last 10 min of each superfusate was recorded and used for analysis. Fictive respiratory-related neural discharges were classified based on previously described criteria, obtained from comparison of the *in vitro* motor output to fictive breathing in the less reduced decerebrate, paralyzed, unidirectionally ventilated *in situ* preparations (Kogo et al., 1994; Sakakibara, 1984; Gdovin et al., 1998). Fictive lung ventilation was defined as high amplitude, low-frequency bursts occurring simultaneously in CN VII, CN X, and CN XII, having an incrementing–decrementing pattern and a duration $< 1 \text{ s}$ (Reid and Milsom, 1998; Torgerson et al., 1998). Fictive gill activity in the tadpole and buccal activity in the adult are characterized by low amplitude high frequency oscillations (Reid and Milsom, 1998; Torgerson et al., 1998). Discharges of motor output not meeting these criteria were assumed to be associated with events other than normal fictive breathing and were excluded from analysis (Hedrick and Winmill, 2003). Burst frequency is defined as neural bursts min^{-1} . Burst duration was measured from the onset of deviation from the baseline to the return to baseline in the integrated neural trace. Burst amplitude was measured in arbitrary units and analyzed as a percentage of control from the integrated neural trace.

Linear regression analysis was used to determine relationships between dependent and independent variables. Data expressed as percentages were arcsine transformed prior to statistical analysis (Zar, 1974). All statistical analyses were carried out using commercially available software programs (Graphpad Prism, v. 3.0.1, San Diego, CA; Igor Pro v. 4.01, Wavemetrics, Inc., Lake Oswego, OR).

RESULTS

All tadpole brainstem–spinal cord preparations ($N = 7$) generated robust rhythmic motor output in the form of low-amplitude, high-frequency fictive gill bursts and high-amplitude, low-frequency fictive lung bursts similar to previous studies (Fig. 1; Gdovin et al. 1998; Torgerson et al. 1998). In the adult, respiratory-related motor output consisted of high-amplitude, low-frequency fictive lung bursts, present in all preparations, and the nonventilatory fictive buccal rhythm, characterized by high-frequency, low-amplitude bursts, observed in 4 of 10 preparations (Fig. 1).

Fictive gill burst frequency was dependent upon $[K^+]_o$ in the range of 1 mM up to 10 mM [Fig. 2(A)]; however, when $[K^+]_o$ was increased to 12 mM, neural activity ceased in all preparations. Figure 2(A) shows integrated neural activity recorded from trigeminal (CN V) and hypoglossal (CN XII) nerve roots in a premetamorphic tadpole. Both gill and lung bursts are readily identifiable in CN V, whereas CN XII contains only lung burst activity. These recordings are very similar to those obtained in previous studies using pre-metamorphic tadpoles (Torgerson et al., 1997, 1998). In premetamorphic tadpoles, CN XII has been used as a “marker” of fictive lung bursts because gill activity is usually not present in the hypoglossal nerve (Torgerson et al., 1998). With increasing $[K^+]_o$, there are clear increases in both gill and lung burst frequencies and at higher levels of $[K^+]_o$ (10 mM), gill burst activity appears in CN XII [Fig. 2(A)]. An example of respiratory-related neural activity recorded in CN XII is shown for an adult preparation in Figure 2(B). Buccal activity was present in 4 of 10 preparations, and an example is shown in Figure 2(B). There is a clear increase in buccal frequency with increasing $[K^+]_o$, but no change in lung burst activity (Table 1).

Frequencies of gill (f_{gill}) and lung (f_{lung}) activity for individual tadpole preparations were plotted against $[K^+]_o$ [Fig. 3(A) and (C)]. Under control conditions ($[K^+]_o = 4 \text{ mM}$) mean f_{gill} was $70.8 \pm 3.4 \text{ min}^{-1}$ while mean f_{lung} was $1.9 \pm 0.3 \text{ min}^{-1}$ (Table 1). Both f_{gill} and f_{lung} were significantly dependent on $[K^+]_o$ [Fig. 3(A) and (C)]. As $[K^+]_o$ was increased from 1 to 10 mM, f_{gill} exhibited an approximate threefold linear increase ($f_{\text{gill}} = 8.6*[K^+]_o + 37.7$; $F(1, 22) = 86.1$; $r^2 = 0.80$; $p < 0.001$) and a concomitant decrease in burst duration (d_{gill}) from $1.23 \pm 0.13 \text{ s}$ at 1 mM to $0.47 \pm 0.05 \text{ s}$ at 8 mM ($d_{\text{gill}} = 1.19 - 0.09*[K^+]_o$; $F(1, 12) = 39.3$; $r^2 = 0.77$; $p < 0.001$) [Fig. 3(A) and B].

Fictive lung burst frequency (f_{lung}) followed a similar $[K^+]_o$ -dependent linear increase similar to that

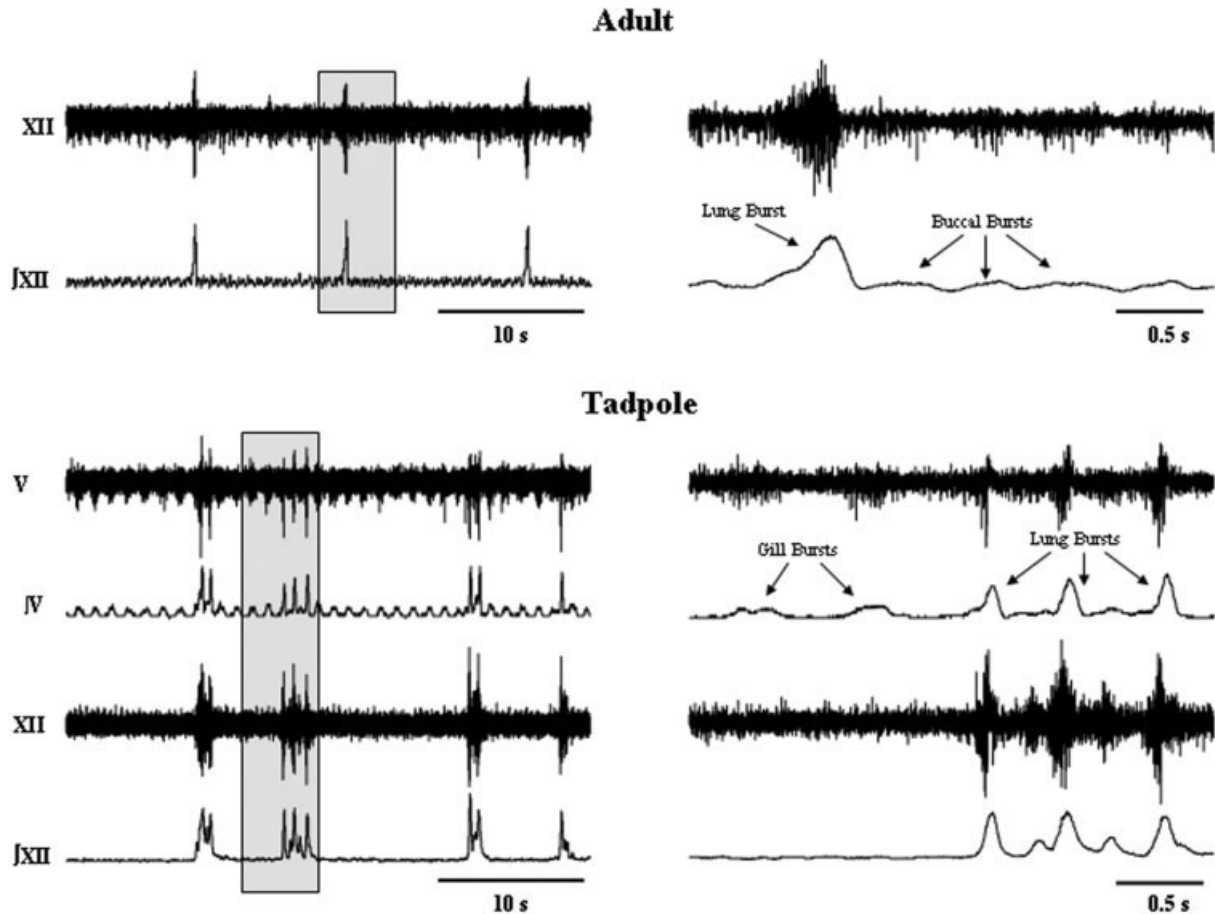


Figure 1 Fictive breathing in isolated adult and larval brainstem preparations. Raw hypoglossal nerve activity (XII) and integrated (f_{XII}) nerve activity in an adult preparation is shown (top). The gray boxed area delineates an expanded portion of the trace on the right showing a single lung burst and several buccal bursts. Raw trigeminal (V) and hypoglossal (XII), and integrated nerve activity (f_V and f_{XII}) from these nerves is shown. An expanded portion of the trace (boxed area) is shown at the right. Note that gill burst activity is present in CN V, but not CN XII in the tadpole.

seen for gill activity $\{f_{lung} = 0.86*[K^+]_o - 1.3; F(1, 22) = 79.4; r^2 = 0.78; p < 0.001\}$ [Fig. 3(C)]. Increasing $[K^+]_o$ to 12 mM stopped respiratory-related bursting in all seven preparations; only tonic activity remained [Fig. 2(A)]. This effect was reversible because both f_{gill} and f_{lung} returned to control levels when preparations were again superfused with control aCSF ($[K^+]_o = 4$ mM). A comparison of f_{gill} during control superfusion before and after changes in $[K^+]_o$ revealed no significant difference (paired t test; $t_4 = 0.2; p > 0.8$). Control lung burst activity (f_{lung}) in tadpoles was similarly unaffected by changes in $[K^+]_o$ (paired t test; $t_4 = 0.7; p > 0.5$). Furthermore, preparations in which $[K^+]_o$ was incrementally increased to 12 mM (4.0, 8.0, 10.0, 12.0 mM) then progressively brought back to control ($N = 2$), showed no hysteresis in the frequency response. Because both f_{gill} and f_{lung} were highly dependent on

$[K^+]_o$, we examined the correlation between $[K^+]_o$ -dependent changes in gill/lung activity. Linear regression revealed a significant relationship between gill and lung burst frequency $\{f_{lung} = 0.80*f_{gill} - 3.5; F(1, 22) = 40.8; r^2 = 0.65; p < 0.001\}$ [Fig. 2(D)]. Increasing $[K^+]_o$ had no significant effect on gill or lung burst amplitude, nor did $[K^+]_o$ alter lung burst duration ($p > 0.1$).

In all adult preparations ($N = 10$), clear lung bursts were present during superfusion with control aCSF ($[K^+]_o = 4$ mM), but buccal activity was present in only 4 of 10 preparations. Lung burst frequency averaged $6.4 \pm 1.0 \text{ min}^{-1}$ in control conditions (Table 1) and did not change significantly with alterations in $[K^+]_o$ up to 8 mM; $F(1, 40) = 2.36; r^2 = 0.06; p > 0.1$ [Fig. 4(A)]. Increasing $[K^+]_o$ to 10 mM resulted in a loss of respiratory-related neural activity [Fig. 2(B)], but this effect was completely reversible be-

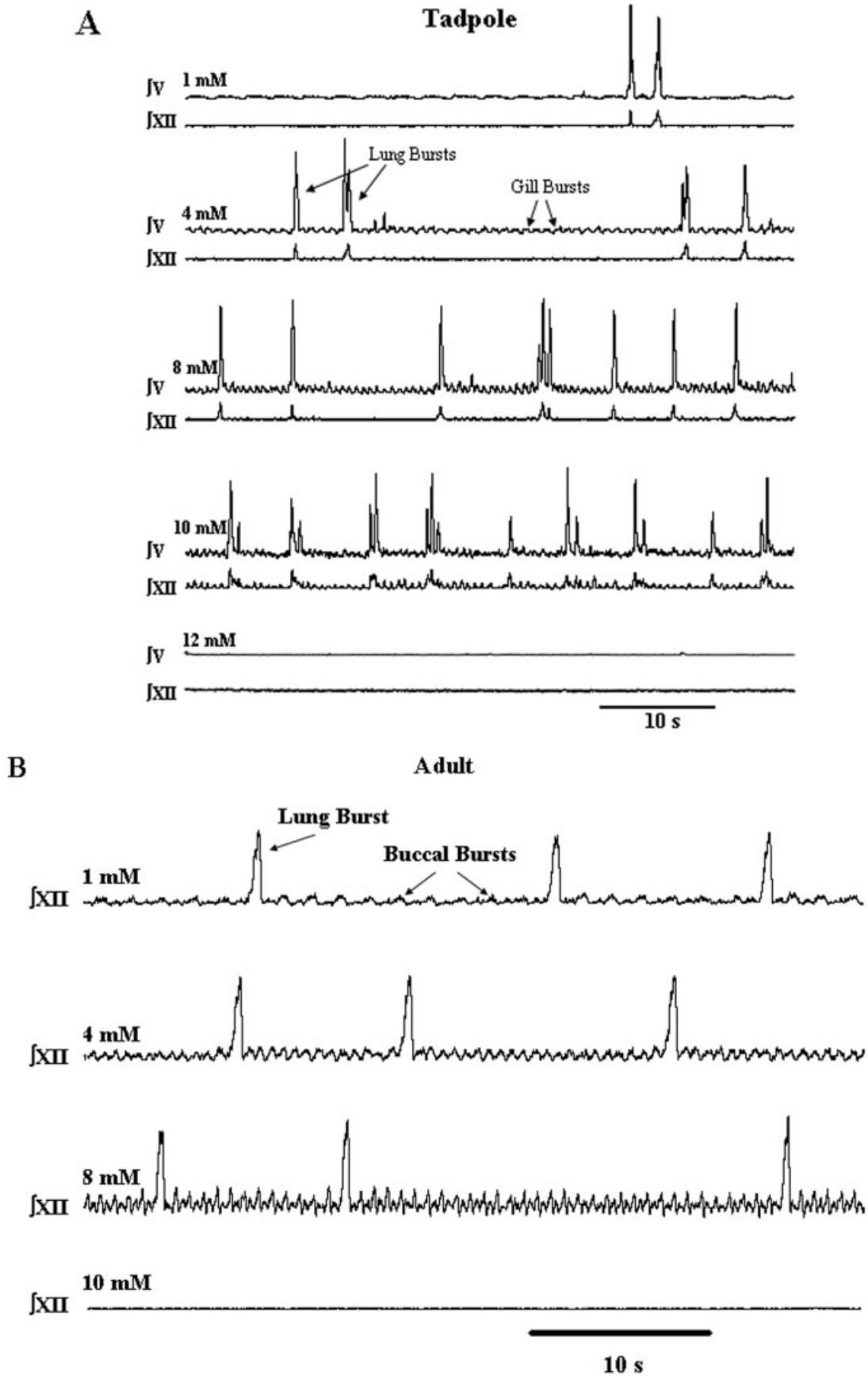


Figure 2

Table 1 Effects of Extracellular K^+ ($[K^+]_o$) (mM) on Fictive Breathing (Bursts min^{-1}) in Tadpole and Adult Bullfrog Brainstem Preparations

	$[K^+]_o$ (mM)			
	1	4	8	10
	Tadpole			
Gill	45.8 ± 11.4 (4)	70.8 ± 3.4 (10)	113.6 ± 4.6 (6)	115.7 ± 5.4 (4)
Lung	0.2 ± 0.1 (4)	1.9 ± 0.3 (10)	4.5 ± 0.6 (6)	8.7 ± 0.8 (4)
	Adult			
Buccal	32.0 ± 1.5 (6)	54.1 ± 6.6 (9)	79.1 ± 4.4 (2)	0
Lung	6.5 ± 2.7 (3)	6.4 ± 1.0 (16)	4.3 ± 1.9 (4)	0

Values are mean ± S.E. (number of observations).

cause f_{lung} during superfusion with control aCSF was not different following changes in $[K^+]_o$ (paired t test; $t_6 = 0.4$; $p > 0.7$). Lung burst amplitude in adults was not affected by changes in $[K^+]_o$ ($p > 0.25$).

Although alterations in $[K^+]_o$ had no effect on lung activity in the adult preparations, fictive buccal frequency (f_{buccal}) was significantly dependent on $[K^+]_o$, exhibiting a linear relationship similar to that observed for f_{gill} and f_{lung} in the tadpole ($f_{\text{buccal}} = 6.6*[K^+]_o + 29.3$; $F(1, 19) = 17.0$; $r^2 = 0.47$; $p < 0.001$) [Fig. 4(B)]. At low $[K^+]_o$ (1 mM), f_{buccal} was $32 \pm 1.5 \text{ min}^{-1}$, compared to a control rate of $54.1 \pm 6.6 \text{ min}^{-1}$ (4 mM), while elevated $[K^+]_o$ (8 mM) increased f_{buccal} to $79.1 \pm 4.4 \text{ bursts min}^{-1}$ (Table 1). Buccal activity was also reversible because f_{buccal} with control aCSF before and after changes in $[K^+]_o$ were not significantly different (paired t test; $t_2 = 0.4$; $p > 0.7$).

Buccal burst duration (d_{buccal}) was significantly reduced with increasing $[K^+]_o$, from an average of $0.82 \pm 0.05 \text{ s}$ at 1 mM to $0.49 \pm 0.04 \text{ s}$ at 8 mM ($d_{\text{buccal}} = 0.88 - 0.04*[K^+]_o$; $F(1, 11) = 9.4$; $r^2 = 0.46$; $p < 0.01$) [Fig. 4(C)]. Changes in $[K^+]_o$ had no significant effect buccal burst amplitude in the adult ($p > .5$). Owing to the lack of effect of $[K^+]_o$ on f_{lung} [Fig. 4(A)], there was no significant correlation between f_{lung} and f_{buccal} in adult preparations, $F(1, 11) = 1.5$; $r^2 = 0.12$; $p > 0.2$) [Fig. 4(D)].

DISCUSSION

This study revealed a developmental change in the effects of $[K^+]_o$ on respiratory rhythm generation in the bullfrog. In the tadpole, both f_{gill} and f_{lung} were highly dependent on $[K^+]_o$, suggesting that both gill and lung burst activities are voltage-modulated. In the isolated adult brainstem, however, although f_{buccal} was significantly dependent on $[K^+]_o$, indicative of a voltage-modulated drive, f_{lung} was unaffected by alterations in $[K^+]_o$. Although these data do not entirely support or refute any particular model for respiratory rhythm generation, the results suggest there is a maturational change in the neural networks involved in respiratory rhythm generation in the amphibian brainstem. These developmental changes suggest that gill and lung ventilation may be highly voltage-dependent in the aquatic tadpole, but lung ventilation becomes largely voltage-independent after metamorphosis to the terrestrial adult stage.

In the tadpole, increasing tonic excitation in the brainstem by altering $[K^+]_o$ one order of magnitude (1 to 10 mM) resulted in a significant, linear increase in f_{gill} and f_{lung} . Fictive gill frequency increased about threefold over this range of $[K^+]_o$ (Table 1), and is comparable to gill ventilation rates *in vivo* for premetamorphic tadpoles at similar stages of development (Burggren and Doyle, 1986). Indeed, the large

Figure 2 Effects of $[K^+]_o$ on fictive breathing in the isolated brainstem preparation of the bullfrog. (A) Integrated $\int V$ and $\int XII$ in the tadpole; f_{gill} and f_{lung} increase with increasing $[K^+]_o$, up to 10 mM and fictive breathing is abolished at 12 mM. Note the appearance of gill burst activity when $[K^+]_o$ is increased to 10 mM. (B) Integrated hypoglossal nerve ($\int XII$) in the adult; f_{buccal} increases with increasing $[K^+]_o$, while f_{lung} is unaffected up to 8 mM and fictive breathing is abolished at 10 mM.

Tadpole

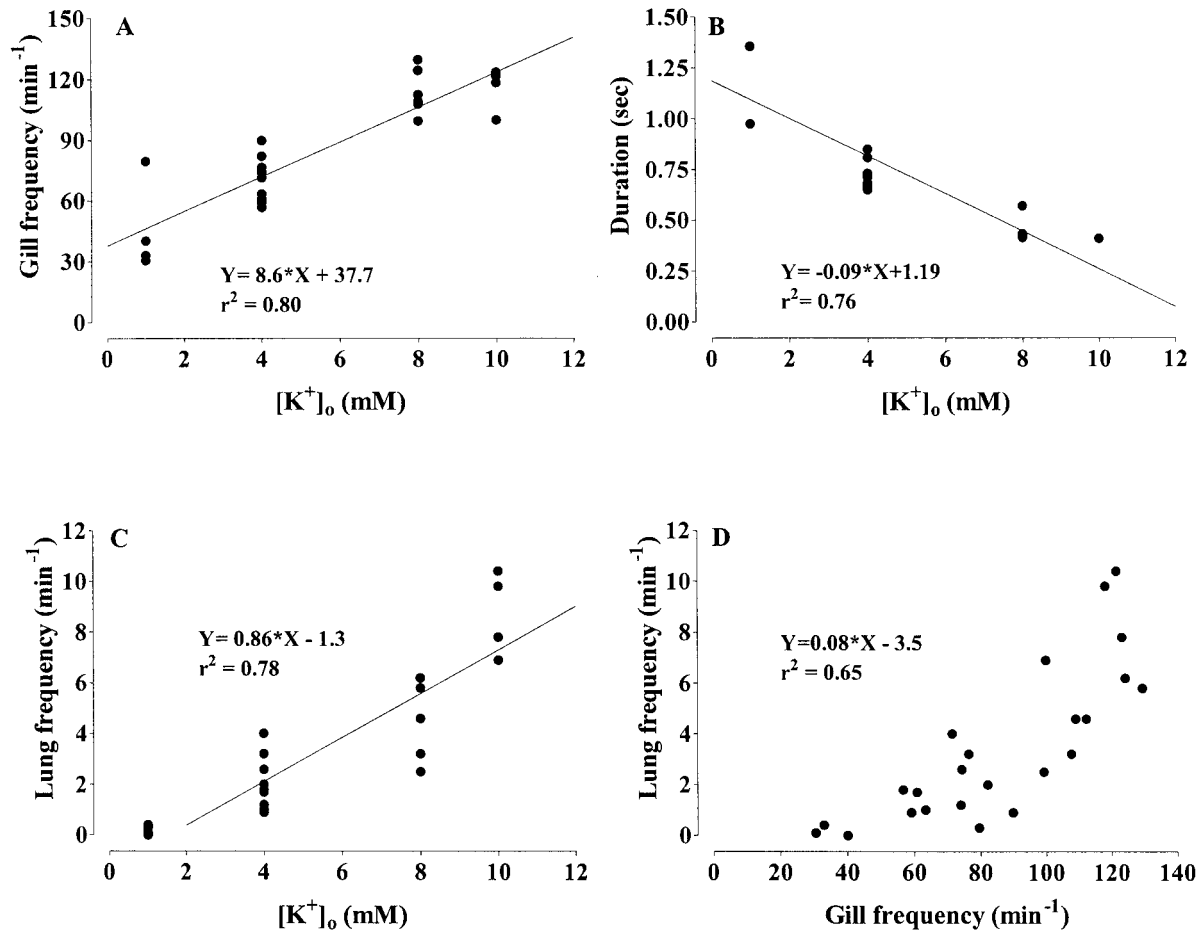


Figure 3 Respiratory burst frequency and burst duration with altered $[K^+]_o$ in the tadpole. (A) Gill burst frequency exhibits a significant, linear increase with changes in $[K^+]_o$; (B) gill burst duration exhibits a linear decrease in burst duration with $[K^+]_o$; (C) fictive lung burst frequency exhibits a linear, $[K^+]_o$ -dependent increase; (D) a significant correlation between $[K^+]_o$ -dependent increases in fictive gill and lung burst frequencies.

increases in f_{gill} and f_{lung} with increasing $[K^+]_o$ are similar to ventilatory changes seen in tadpoles *in vivo* from rest to exercise (Burggren and Doyle, 1986). The effects of $[K^+]_o$ on respiratory-related activity were completely reversible since respiratory activity was identical before and after changes in $[K^+]_o$ and there was no apparent hysteresis in the frequency response to changes in $[K^+]_o$ (see Results).

Fictive lung bursts in premetamorphic tadpoles in this study under control conditions were somewhat higher than lung ventilation rates *in vivo* at comparable stages of development (Burggren and Doyle, 1986; Crowder et al., 1998). Although lungs are present and lung ventilation is observed at all developmental stages, lung ventilation is infrequent, with gills and skin supplying the major routes of oxygen

uptake and carbon dioxide elimination (Burggren and Doyle, 1986; Crowder et al., 1998). Recent observations in *Rana catesbeiana* indicate that obligate air breathing does not begin until regression of the gills (ca. T-K stages XXII–XXIII), and there is no correlation between lung ventilation and developmental stage up to the point of metamorphosis (Crowder et al., 1998). Mean lung ventilation rates *in vivo* are about one to six breaths h^{-1} , but there is considerable variability in this behavior (Crowder et al., 1998). One explanation for the higher lung ventilation rates *in vitro* is that it appears that the neural networks supporting lung bursts are present, but suppressed by GABA_B -ergic inhibition, at early stages of development (Strauss et al., 2000). The progressive unmasking of a “silent,” but functional, rhythmic pattern

Adult

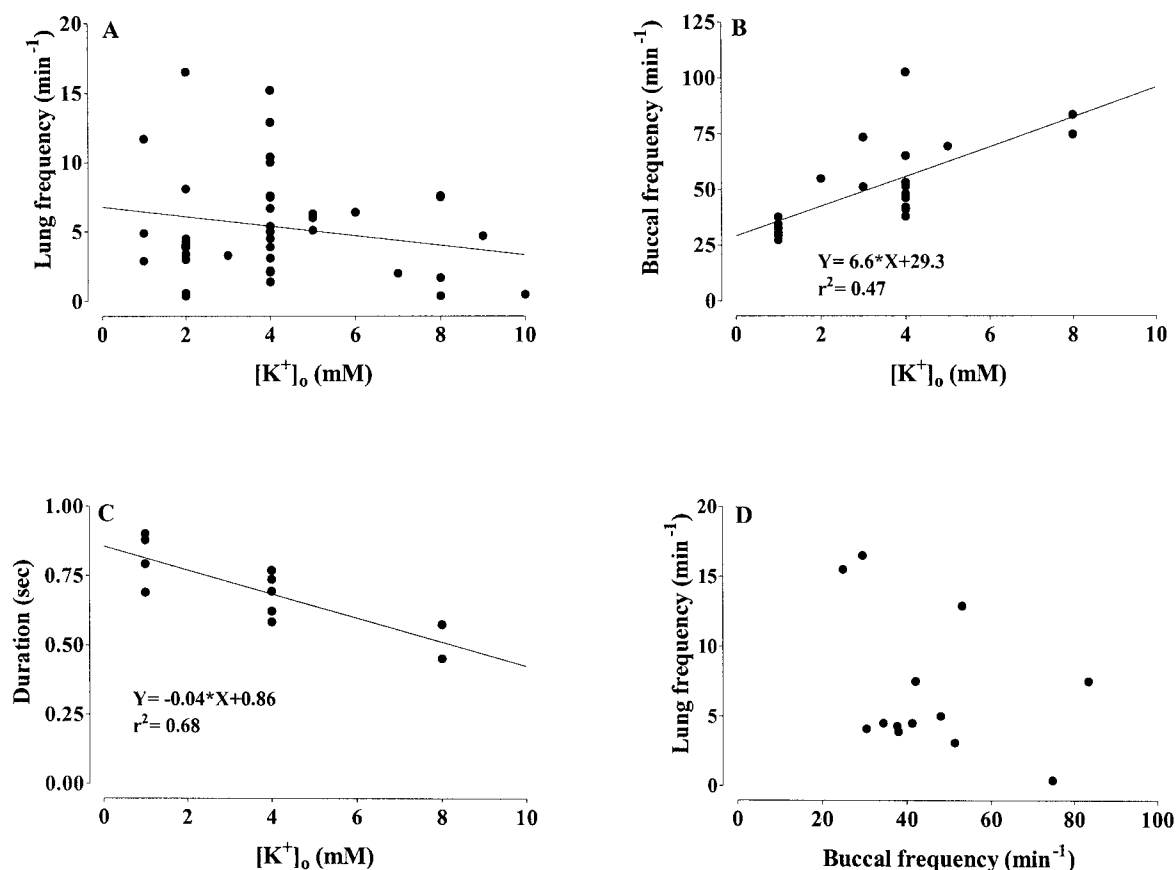


Figure 4 Respiratory burst frequency and characteristics with altered $[K^+]_o$ in the adult isolated bullfrog brainstem. (A) Lung burst frequency in the adult is unaffected by alterations in $[K^+]_o$; (B) buccal burst frequency exhibits a significant linear, $[K^+]_o$ -dependent increase in burst frequency; (C) buccal burst duration exhibits a linear decrease in burst duration; (D) there was no correlation between lung burst frequency and buccal burst frequency.

generator in early developmental stages by removal of inhibitory inputs is a common feature in the assembly of adult central pattern generators (Fenelon et al., 1998). Lower lung ventilation rates *in vivo* may reflect a central or peripheral inhibition of an intrinsically higher rate produced by brainstem respiratory networks. The increase of lung burst frequency *in vitro* with increasing $[K^+]_o$ may also support this concept if it can be shown that a progressive depolarization of respiratory neurons occurs during development of respiratory rhythm generation. However, testing this hypothesis would require intracellular measurements from respiratory neurons in tadpoles at different stages of development.

Increases in $[K^+]_o$ raises the general excitability of cells by membrane depolarization, and this would be expected to have diverse effects on respiratory neurons (see Richter and Spyer, 2001). This would in-

clude the opening of a number of voltage-gated and K^+ -dependent ion channels, and voltage-dependent neurotransmitter release. Any of these may affect respiratory networks or motoneurons. We have assumed that increasing $[K^+]_o$ depolarizes all respiratory and nonrespiratory neurons, but there may be heterogeneous responses to this depolarization. For example, there are likely different resting potentials of neurons and a variety of K^+ channels that would respond differently to this stimulus. In the present study, tadpole preparations in which $[K^+]_o$ was incrementally increased from control (4 mM) to 12 mM and then progressively brought back to control, showed no hysteresis in $[K^+]_o$ -dependent changes respiratory output. Thus, it seems unlikely that depolarization-evoked neurotransmitter release is involved in the responses observed in this study. It is possible that maturational changes in membrane potential or

ion channel expression in the respiratory networks of larval and adult bullfrogs may underlie the differential effects of $[K^+]_o$ on fictive breathing. In mammals, the membrane potential of neonatal respiratory and pacemaker neurons becomes increasingly hyperpolarized with development (Richter and Spyer, 2001). Many of the ion channels expressed and functioning during rhythm generation in the adult mammal *in vivo* are expressed, but do not appear to be functional at birth and are not essential for pacemaker activity in the neonatal respiratory network (Richter and Spyer, 2001).

Because both gill and lung burst activities in the tadpole were highly dependent upon $[K^+]_o$, our data do not allow us to distinguish between pacemaker-driven and network-driven processes for respiratory neural activities. Both pacemaker models and neural network models for respiratory rhythm generation contain neurons with voltage-dependent properties (Butera et al., 1999a, 1999b; Smith et al., 2000; Del Negro et al., 2001; Rybak et al., 2002). An important distinction between our experiments and those from mammalian brainstem slices *in vitro* is that fictive breathing in tadpole and adult brainstem preparations occurs spontaneously at normal $[K^+]_o$ (4 mM), and over a broad range of $[K^+]_o$ (1–10 mM), whereas fictive breathing in mammalian brainstem slices occurs only when $[K^+]_o$ is increased beyond the normal range (Koshiya and Smith, 1999; Johnson et al., 2001).

Network plasticity and production of different motor outputs by a single pattern generator is common (Marder and Bucher, 2001), but the bulk of evidence in amphibians suggests that anatomically distinct neural networks with different mechanisms generate respiratory activity in tadpoles and adults. Chloride-mediated synaptic inhibition is essential for generating gill ventilation in tadpoles, but pacemaker-like activity appears to drive lung ventilation (Galante et al., 1996; Broch et al., 2002). In the adult bullfrog, however, synaptic inhibition is important for generating lung burst activity (Broch et al., 2002). In addition, brainstem transections and microinjection of glutamatergic antagonists into discrete brainstem areas of tadpoles also support the hypothesis that neural networks generating gill/buccal and lung ventilation in pre- and postmetamorphic tadpoles are anatomically distinct (Torgerson et al., 2001; Wilson et al., 2002).

Based upon previous experiments and the present study, we hypothesize that lung ventilation in bullfrogs matures from voltage-dependent, pacemaker-driven process in the tadpole to a voltage-independent, network-driven process in the adult. Overall, the results of this study are consistent with studies from

neonate and adult mammals, suggesting that there are significant developmental changes in the mechanisms of respiratory rhythm generation (Richter and Spyer, 2001).

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