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Regulation of the respiratory central pattern generator by chloride-dependent inhibition during development in the bullfrog (*Rana catesbeiana*)

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Summary

Isolated brainstem preparations from larval (tadpole) and adult *Rana catesbeiana* were used to examine inhibitory mechanisms for developmental regulation of the respiratory central pattern generator (CPG). Preparations were superfused at 20–22 °C with Cl^--free artificial cerebrospinal fluid (aCSF) or with aCSF containing agonists/antagonists of γ-aminobutyric acid (GABA) or glycine receptors. Respiratory motor output from the CPG, measured as neural activity from cranial nerve roots, was associated with fictive gill ventilation and lung ventilation in tadpoles and with fictive lung ventilation in adults. In tadpoles, fictive lung burst frequency was 0.8± 0.2 min^{-1} and did not change significantly with Cl^--free aCSF superfusion; however, lung burst amplitude increased by nearly 400 % (P<0.01). Fictive gill ventilation averaged 41.6±3.3 min^{-1} and was reversibly abolished by Cl^--free aCSF. Superfusion with Cl^--free aCSF abolished lung bursts in two of seven adult preparations, and overall lung burst frequency decreased from 3.1±0.7 to 0.4±0.03 min^{-1} (P<0.01), but burst amplitude was unchanged. Low concentrations of GABA (0.5 mmol l^{-1}) produced a significant increase in lung burst frequency followed by almost complete inhibition at 5.0 mmol l^{-1}, accompanied by the abolition of gill ventilation at 2.5–5.0 mmol l^{-1}. By contrast, fictive lung ventilation in adults was inhibited in a dose-dependent manner by glycine and GABA, and inhibition occurred at approximately 10-fold lower concentrations compared with tadpoles. The glycine receptor antagonist strychnine (2.5–25.0 μmol l^{-1}) and the $GABA_A$ receptor antagonist bicuculline (1–10 μmol l^{-1}) inhibited fictive gill ventilation and increased fictive lung ventilation in tadpoles. However, bicuculline and strychnine inhibited fictive lung ventilation in adults. These results suggest that lung ventilation in the tadpole brainstem may be driven by a pacemaker-like mechanism since Cl^--free aCSF failed to abolish lung ventilation. Lung ventilation in adults and gill ventilation in tadpoles, however, appear to be dependent upon conventional Cl^--mediated synaptic inhibition. Thus, there may be a developmental change in the fundamental process driving lung ventilation in amphibians. We hypothesize that maturation of the bullfrog respiratory CPG reflects developmental changes in glycinergic and/or GABAergic synaptic inhibitory mechanisms.

Key words: amphibian, bullfrog, *Rana catesbeiana*, central pattern generator, fictive breathing, GABA, glycine, strychnine, bicuculline.

Introduction

The ontogenetic transition from an aquatic to a terrestrial habitat in amphibians is accompanied by major maturational changes in the respiratory system, including a shift from gill to lung ventilation (Burggren and West, 1982; Gdovin et al., 1999), morphological changes associated with the change from aquatic to aerial respiration (Gradwell, 1972; Burggren and Doyle, 1986) and the development of central CO_2/H^+ receptors (Torgerson et al., 1997b, 2001a). Underlying many of these changes are alterations in the anatomical substrates, neural connections and modulation associated with the neural circuits, or central pattern generators (CPGs), that drive breathing in amphibians and other vertebrates (Torgerson et al., 1998, 2001b; Gdovin et al., 1999).

In larval amphibians, gas exchange is accomplished primarily by rhythmic ventilation of the gills, but as development progresses lung ventilation assumes a greater fraction of overall gas exchange (Burggren and West, 1982). Recent evidence suggests that there is a ‘translocation’ of the primary site(s) for neural generation of lung ventilation from the caudal brainstem to the rostral brainstem in developing tadpoles (Torgerson et al., 2001b). Upon metamorphic climax, gill ventilation is replaced by rhythmic buccal movements characteristic of adults, with lung ventilation occurring episodically. Previous work with tadpole brainstem preparations *in vitro* indicates that Cl^--dependent synaptic inhibition may be important for gill ventilation, but not lung ventilation, and that lung ventilation may be driven by a pacemaker-like mechanism (Galante et al., 1996) similar to

that proposed for neonatal mammals (Smith et al., 1991). Furthermore, both GABAergic and glycinergic mechanisms may be important modulators of the amphibian CPG in tadpoles (Galante et al., 1996; Strauss et al., 2000). However, the extent to which $GABA_A$ or glycinergic pathways contribute to age-dependent inhibition of ventilation in amphibians is unclear.

There is emerging evidence that the mechanisms regulating central respiratory rhythmogenesis and respiratory motor output in amphibians share many common features with those of developing mammals (Gdovin et al., 1999). Thus, amphibians appear to be excellent models for examining the development of respiratory rhythm generation. The amphibian brainstem/spinal cord preparation is particularly well suited as a developmental model for examining the mechanisms of respiratory rhythm generation because the gamut of stages from tadpole to adult frog can be studied using identical experimental techniques at all stages of development.

In this study, we tested the hypothesis that aquatic-to-terrestrial development in amphibians is associated with the development of Cl^--dependent synaptic inhibitory mechanisms for respiratory rhythm generation. For this purpose, we used isolated brainstem/spinal cord preparations from larval and adult North American bullfrogs (*Rana catesbeiana*).

Materials and methods

Animals

Experiments were performed on 30 larval (body mass 10.4±0.9 g) and 37 adult (body mass 127±5.4 g) North American bullfrogs (*Rana catesbeiana*). The tadpoles were in developmental stages XIV to XIX based on the criteria established for *Rana pipiens* (Taylor and Köllros, 1946). Animals were obtained from commercial suppliers (tadpoles, Charles Sullivan, Inc., Nashville, TN, USA; adults, West Jersey BioServices, Inc., Wenonah, NJ, USA). The adult frogs were maintained in plastic tanks with continuous access to water; tadpoles were kept in aquaria with aerated, dechlorinated tapwater. All animals were maintained at room temperature (20–22 °C).

Surgical procedures

Adult frogs and tadpoles were initially anesthetized in an aqueous solution of tricaine methanosulfonate (MS222; 0.5 g l^{-1} for tadpoles and 1.5 g l^{-1} for adult bullfrogs) buffered to pH 7.4 with $NaHCO_3$. When breathing movements ceased (2–4 min for tadpoles and 10–20 min for adult frogs), the animals were quickly removed and placed in ice for 1 h to maintain anesthesia and reduce metabolic rate. Following this, the brain was exposed and the forebrain rostral to the optic lobes was transected. Throughout the decerebration and subsequent dissection, the brainstem was perfused continuously with ice-cold (5–7 °C) artificial cerebrospinal fluid (aCSF) of the following composition (in mmol l^{-1}): 40 $NaHCO_3$, 1.0 NaH_2PO_4, 75 NaCl, 4.5 KCl, 1.0 $MgCl_2.6H_2O$, 7.5 glucose and 2.5 $CaCl_2$ (Hedrick and Morales, 1999) equilibrated with 100 % oxygen. The cranial nerves on both sides were carefully dissected close to the exit from the skull. The entire dissection required approximately 30 min to complete. The isolated brainstem was transferred to a Sylgard-lined recording chamber (7 ml). The brainstem was placed ventral side up, and the dura were removed. Throughout this process, and in all subsequent experiments, the recording chamber was continuously perfused with aCSF (20–22 °C) equilibrated with 98 % O_2 and 2 % CO_2 (pH 7.5–7.6) at a flow rate of 5–10 ml min^{-1}.

Nerve recordings

Nerve recordings were obtained with glass suction electrodes connected to cranial nerve roots V (trigeminal), X (vagus) and XII (hypoglossal) in the isolated adult frog brainstem and to cranial nerve roots V, VII (facial) and XII in the tadpole preparation. The electrodes were connected to a differential alternating current amplifier (A-M systems, model 1700), and action potentials were amplified 10 000-fold, filtered (10 Hz to 5 kHz) and moving time averaged (CWE model MA-821-4). Raw and processed signals were simultaneously recorded on computer with a data-acquisition system sampling at 2 kHz (MacLab 8S; AD Instruments) and on videotape with pulse code modulation (Vetter PCM model 402).

Superfusion with Cl^--free aCSF

Ten tadpoles and seven adult bullfrogs were used for experiments in which isolated brainstem preparations were superfused with Cl^--free aCSF. To test the importance of Cl^--mediated inhibition for respiratory rhythmogenesis in the isolated brainstem, Cl^- was replaced with the anion salts of gluconic acid (Cl^--free aCSF). The Cl^--free aCSF had the following composition (in mmol l^{-1}): 40 $NaHCO_3$, 1.0 NaH_2PO_4, 75 sodium gluconate, 4.5 potassium gluconate, 1.0 magnesium gluconate, 7.5 glucose and 2.5 calcium gluconate.

The recording chamber was connected to two identical parallel reservoirs from which the brainstem was superfused with control aCSF or with Cl^--free aCSF by switching between the two reservoirs. CO_2 content was adjusted to maintain aCSF pH between 7.5 and 7.6 at all times. In each experiment, the control recording was followed by a 20 min period of superfusion with Cl^--free aCSF. The last 10 min of recorded data was used for analysis.

Superfusion with agonists and antagonists of GABA and glycine receptors

Preliminary experiments were performed to determine the minimal and maximal effects on respiratory-related activity of the agonists and antagonists of γ-aminobutyric acid (GABA) and glycine receptors. The following concentration ranges were used: GABA and glycine (0.1–5.0 mmol l^{-1}), bicuculline (1–10 μmol l^{-1}) and strychnine (2.5–25.0 μmol l^{-1}). The solutions were freshly prepared in aCSF before each experiment and adjusted to pH 7.5–7.6 while bubbling with 98 % O_2/2 % CO_2. The control recording was followed by a

15 min brainstem superfusion with aCSF followed by progressively increasing concentrations of the agonist or antagonist achieved by switching between the two reservoirs connected to the recording chamber. The last 10 min of each drug superfusion period was recorded and used for analysis. After superfusion with the highest concentration of the drug, a washout period of 30–180 min with aCSF was necessary to re-establish control conditions, depending on the drug used. In most cases, a single drug was used per preparation, but in five tadpole preparations, two different drugs (agonist followed by antagonist) were used per preparation.

Data analysis and statistics

Previous studies have established the correlation between the mechanical events and neural activity associated with lung ventilation for adult (Sakakibara, 1984) and larval (Gdovin et al., 1998) bullfrogs. Thus, neural activity associated with gill and lung ventilation in tadpoles, and lung ventilation in adults, has been clearly established *in vivo* and used to identify fictive gill and lung bursts *in vitro* (McLean et al., 1995; Galante et al., 1996; Torgerson et al., 1998). In adult bullfrogs, neural activity associated with lung ventilation occurs as single breaths or as an episodic series of breaths. Although episodic breathing does occur *in vitro*, it is not present in every preparation and lung bursts often occur as single breaths (Reid and Milsom, 1998). For analysis, we did not distinguish between single breaths or episodic breathing. Lung burst frequency was measured as the total number of breaths divided by the total recording period, which was normally 10–15 min. Lung burst duration was measured from the onset at the baseline to the termination at the baseline from the integrated neural trace, and lung burst amplitude was measured in arbitrary units from the integrated neural signal.

In tadpole brainstem preparations, single fictive lung bursts were distinguished from fictive gill bursts as large-amplitude bursts recorded simultaneously in cranial nerves VII and XII. Burst activity in cranial nerve XII in tadpoles serves as a 'marker' for lung ventilation *in vivo* (Gdovin et al., 1998) and *in vitro* (Torgerson et al., 1998) because fictive gill ventilation is present in cranial nerves V, VII and X, but not in cranial nerve XII in pre-metamorphic tadpoles. In post-metamorphic tadpoles, such as those used in this study, fictive gill ventilation begins to emerge in cranial nerve XII, but is clearly distinguishable from fictive lung bursts (Torgerson et al., 1998). Fictive gill ventilation is characterized by low-amplitude, high-frequency neural activity compared with the high-amplitude, low-frequency lung bursts (Torgerson et al., 1998).

For each experiment, at least 10 fictive breaths (if present) associated with either gill or lung ventilation were analyzed. Mean values for gill and lung motor output for each preparation were used to calculate the group mean ± S.E.M. A one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (Zar, 1974) was used for evaluation of statistical significance between fictive breaths during drug administration compared with the control within each experimental group. Percentage data were converted to their arcsine values prior to statistical analysis (Zar, 1974). The minimal level of statistical significance was taken as $P<0.05$. Statistical analyses were carried out using commercially available software (GraphPad Prism v. 2.0).

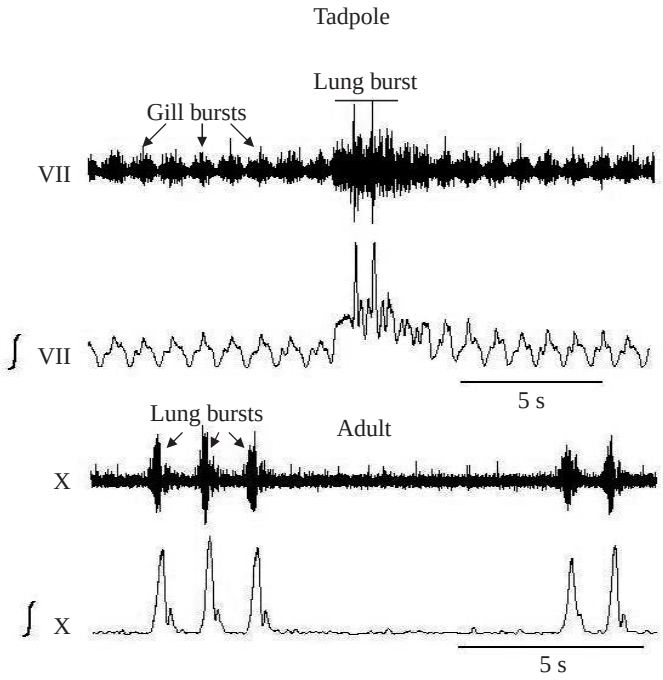

Fig. 1. Representative recordings of fictive breathing from tadpole and adult brainstem preparations. Raw and integrated (∫) neural outputs from cranial nerve VII are shown for a tadpole exhibiting fictive gill breathing and a lung burst. Raw and integrated neural outputs from cranial nerve X are shown for an adult exhibiting fictive lung bursts.

Results

Effects of Cl^--free aCSF

Typical neural activity recorded from tadpole and adult preparations in control conditions is shown in Fig. 1. Fictive gill bursts were robust and present in every tadpole preparation. Activity in adult preparations consisted of lung-related neural bursts that were present as single bursts (Fig. 1) or as episodic bursts (see Reid and Milsom, 1998). Non-ventilatory buccal oscillations, consisting of high-frequency, low-amplitude neural bursts, were infrequent in adult preparations.

A summary of the effects of Cl^--free aCSF superfusion on fictive lung bursts in tadpole and adult brainstem preparations is shown in Table 1. Fictive lung burst frequency in tadpole brainstem preparations was 0.8±0.2 min^{-1} in aCSF and increased slightly (to 1.1±0.1 min^{-1}; $P>0.05$) with Cl^--free superfusion. Although lung burst frequency in tadpoles was unchanged in Cl^--free aCSF, the characteristics of the lung bursts were altered. Individual lung bursts changed from an augmenting burst consisting of several smaller 'peaks' superimposed on the lung burst (Fig. 1) to a single, large-amplitude decrementing burst

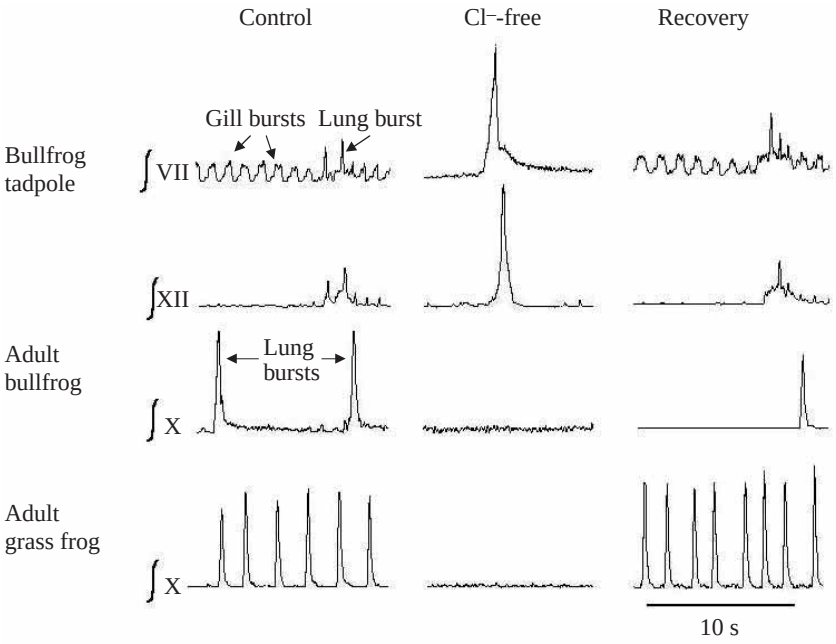

Fig. 2. Effects of Cl^--free artificial cerebrospinal fluid (aCSF) superfusion on integrated (∫) whole-nerve activity recorded from isolated brainstem preparations from a bullfrog tadpole exhibiting fictive gill and lung bursts in cranial nerves VII and XII and from cranial nerve X of an adult bullfrog and an adult grass frog showing fictive lung bursts. Results are shown during superfusion with aCSF (Control), following a 20 min perfusion with Cl^--free aCSF and after a 90 min washout with normal aCSF.

with no small 'peaks' (Fig. 2). For the tadpole preparations, there were 5.8±1.6 peaks burst^{-1}, and this decreased significantly (P<0.05) to 1.03±0.03 peaks burst^{-1} in response to Cl^--free superfusion. Following recovery in normal aCSF, the number of peaks per burst returned to control levels. The amplitude of the lung bursts increased nearly fourfold in response to Cl^--free aCSF superfusion (Table 1; P<0.01) compared with control bursts. Lung burst duration was 8.3±2.6 s in control conditions, measured from cranial nerve XII, and did not change significantly during Cl^--free aCSF superfusion (Table 1).

Fictive gill burst frequency in tadpoles averaged 41.6±3.3 min^{-1} with control aCSF, which is similar to that recorded *in vivo* for tadpoles at similar developmental stages (Burggren and West, 1982; Burggren and Doyle, 1986; Gdovin et al., 1998). Superfusion with Cl^--free aCSF completely abolished gill burst activity in every preparation (Table 1), but activity recovered when the preparation was re-perfused with control aCSF, albeit at a slightly lower frequency (32.4±4.4 min^{-1}; P>0.05).

In adult bullfrog preparations, fictive lung bursts were completely abolished in two of seven experiments in response to Cl^--free aCSF. In the remaining five preparations, lung-related bursts were infrequent and did not resemble normal lung bursts because of their very long duration (Table 1) compared with control bursts. Lung burst frequency decreased significantly from 3.1±0.7 to 0.4±0.03 min^{-1} (P<0.01, N=7) in Cl^--free aCSF (Table 1), but burst amplitude did not change significantly with Cl^- substitution. To determine whether the effects of Cl^--free aCSF were species-specific, and unrelated to developmental age, we examined the effects of Cl^--free aCSF superfusion on brainstem preparations from six adult grass frogs (*Rana pipiens*). Similar to the results with adult bullfrogs, Cl^--free aCSF abolished or significantly reduced lung burst frequency in adult grass frogs (Fig. 2). Overall, fictive lung burst frequency decreased from 19.4±0.8 to 0.1±0.03 min^{-1} (P<0.001; N=6) with Cl^--free aCSF and recovered fully to control values (15.8±4.0 min^{-1}) with normal aCSF.

Effects of GABA and glycine

Tadpole preparations exhibited a biphasic frequency response to GABA, with an initial increase in lung burst frequency at 0.5 mmol l^{-1} GABA followed by a significant inhibition of burst frequency at 5.0 mmol l^{-1} GABA (Fig. 3A). Overall, lung burst frequency increased significantly from 2.6±0.4 to 6.5±1.1 min^{-1} at 0.5 mmol l^{-1} and decreased to 0.1±0.1 min^{-1} at higher concentrations (Fig. 3A). At the highest concentration, lung burst activity was abolished in five of six tadpole preparations. Tadpole brainstem preparations were relatively insensitive to glycine, which had no effect on fictive lung burst frequency except at the highest concentration

Table 1. *Summary of the effects of superfusion with Cl^--free artificial cerebrospinal fluid on ventilatory burst activity in* Rana catesbeiana *tadpole and adult brainstem preparations at 20–22 °C*

	Tadpole (N=10)			Adults (N=7)		
	Control	Cl^--free	Recovery	Control	Cl^--free	Recovery
Lung burst frequency (min^{-1})	0.8±0.2	1.1±0.1	0.4±0.2	3.1±0.7	0.4±0.03*	2.6±0.7
Lung burst duration(s)	8.3±2.6	10.4±3.0	11.9±3.0	1.4±0.3	14.2±5.0*	2.1±0.9
Lung burst amplitude (% control)	100	382±52*	194±63	100	115±19	87±12
Gill burst frequency (min^{-1})	41.6±3.3	0*	32.4±4.4			

Values are means ± S.E.M.
*Significantly different from the control value (P<0.01).

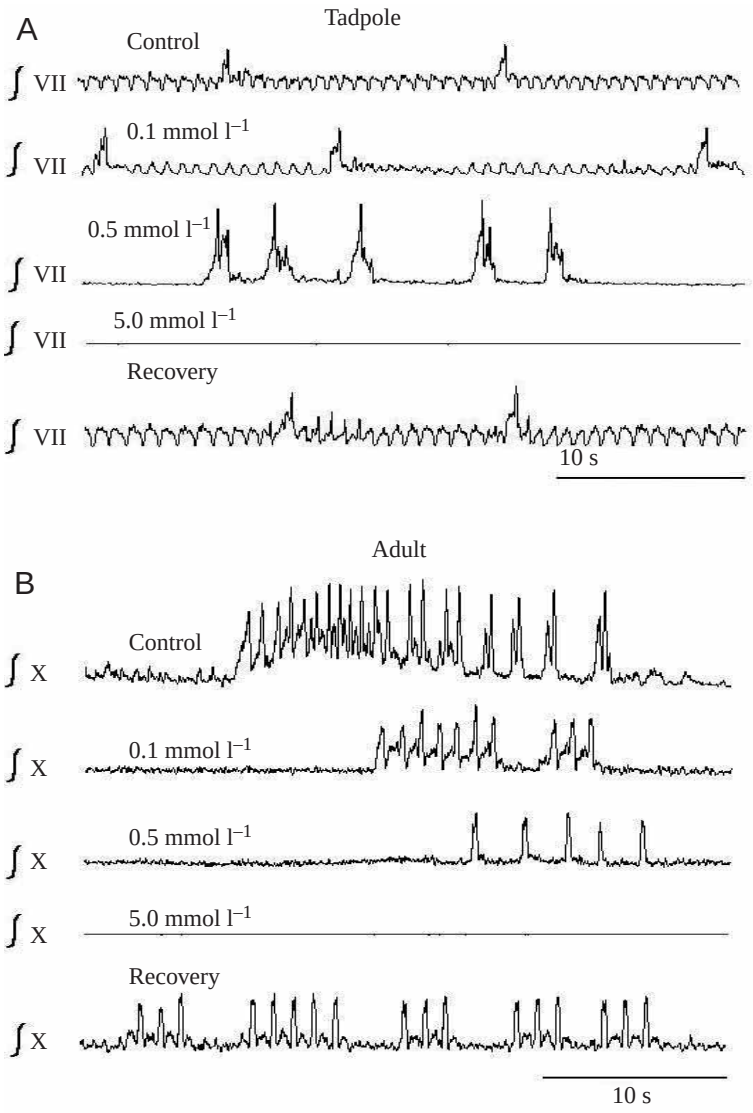

Fig. 3. Effects of increasing concentrations of GABA (from 0.1 to 5.0 mmol l^{-1}) on integrated (∫) neural activity from (A) a tadpole (cranial nerve VII) and (B) an adult (cranial nerve X) brainstem preparation.

used (5.0 mmol l^{-1} glycine), which abolished lung bursts in every preparation.

Fictive gill ventilation was more sensitive to the effects of GABA and glycine than were lung bursts. Gill burst frequency was significantly reduced at lower concentrations of both agonists (0.5 mmol l^{-1}) and completely abolished at higher concentrations (Fig. 3A; glycine data not shown). Both gill and lung bursts returned to control levels after recovery in normal aCSF.

Lung burst activity in adult preparations exhibited a greater sensitivity to GABA and glycine compared with tadpole preparations; that is, significant effects on burst frequency occurred at lower concentrations of the agonists compared with tadpole preparations. In response to superfusion with GABA or glycine, lung burst frequency in adult preparations decreased significantly from approximately 5 to 8 min^{-1} (control) to approximately 1–2 min^{-1} with 0.5 mmol l^{-1} agonist (P<0.05, Figs 3B, 4). Increasing the concentration of GABA or glycine to 5.0 mmol l^{-1} caused further reductions in, or completely abolished, lung burst activity.

Effects of bicuculline and strychnine

For tadpole preparations, superfusion with bicuculline and strychnine, antagonists of $GABA_A$ and glycine receptors, respectively, caused significant increases in lung burst frequency at concentrations of 5.0 μmol l^{-1} (Figs 5A, 6). By contrast, fictive gill bursts were abolished at the same concentrations of either antagonist (Fig. 5A; bicuculline data not shown). Both antagonists significantly increased lung burst amplitude and duration at concentrations of 5–10 μmol l^{-1} in tadpoles: bicuculline increased lung burst amplitude to 138.1±15.1 % of the control value (P<0.05), while strychnine increased the amplitude to 264.3±16.6 % of the control value (P<0.01).

In contrast to tadpoles, bicuculline and strychnine significantly decreased burst frequency in adults (Figs 5B, 6). At 5.0 μmol l^{-1} bicuculline, frequency decreased from 14.0±6.1 to 1.9±0.5 min^{-1} (P<0.01), with no further decline in lung burst activity at higher concentrations. Strychnine produced similar effects on lung burst frequency, reducing lung bursts from 10.1±3.0 to 4.1±0.8 min^{-1} (P<0.05) at 5.0 μmol l^{-1} and further reducing frequency to 2.3±0.5 min^{-1} at 25.0 μmol l^{-1} (Fig. 6). Both antagonists had significant effects on burst shape, changing lung bursts from augmenting to decrementing while also increasing burst duration (Fig. 5B). Lung burst duration was typically approximately 1 s in adults, but increased to 5–8 s in the presence of bicuculline or strychnine. Neither bicuculline nor strychnine had a significant effect on lung burst amplitude in adult preparations.

Discussion

The major findings of this study are (i) that perfusion of isolated brainstem preparations *in vitro* with Cl^--free aCSF abolished, or reduced, lung burst activity in adult bullfrogs and abolished gill bursts in tadpoles, but had no effect on lung burst frequency in tadpoles; (ii) that low concentrations of GABA increased lung burst frequency in tadpoles, but higher concentrations of GABA or glycine reduced lung burst frequency in both tadpole and adult preparations; inhibition occurred at approximately 10-fold lower concentrations in adults compared with tadpoles; and (iii) that the $GABA_A$ receptor antagonist bicuculline and the glycine receptor antagonist strychnine increased lung burst frequency in tadpoles, but decreased lung burst frequency in adults. Collectively, these results argue for a significant developmental change in the role of Cl^--mediated synaptic

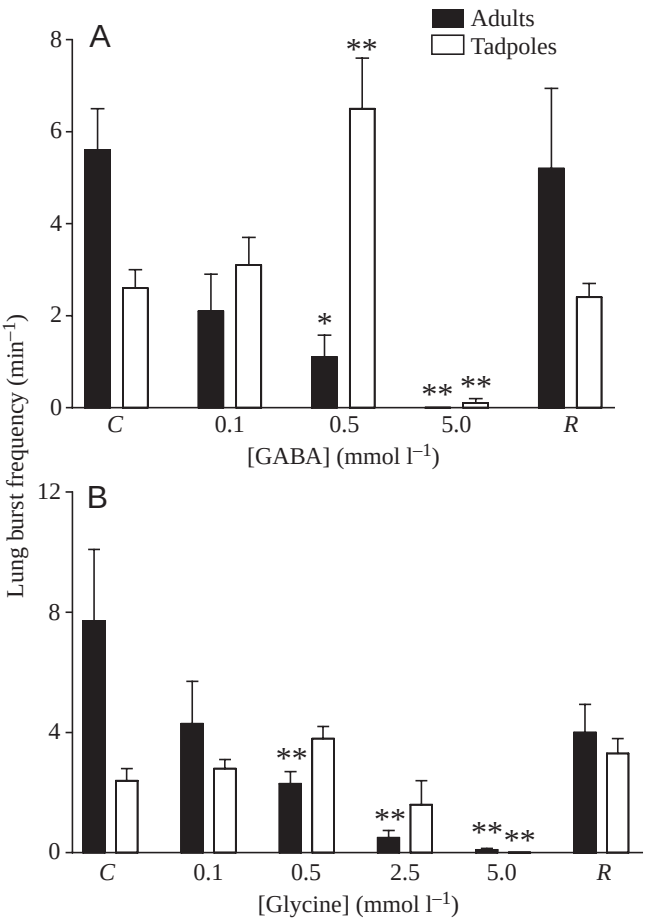

Fig. 4. Summary of the effects of (A) GABA and (B) glycine on fictive lung burst frequency (min^{-1}) in tadpoles (N=6) and adults (N=7). Values are means + S.E.M. *P<0.05, **P<0.01 compared with the control value. *C*, control value; *R*, recovery value.

inhibition through GABAergic and glycinergic inputs to the anuran respiratory CPG.

Critique of methods

The advantages of *in vitro* brainstem/spinal cord preparations in mammals for studying the cellular physiology of breathing are well recognized (Feldman and Smith, 1989). The high metabolic rate of mammalian brain tissue, coupled with the lack of hypoxia-tolerance, has necessitated that studies using *in vitro* preparations be limited to neonatal brainstem preparations to avoid diffusion limitations inherent in these preparations. Mammalian brainstem preparations must also be maintained at lower than normal temperatures to maintain viability, and whole brainstem/spinal cord preparations *in vitro* have been shown to become severely hypoxic and acidic (Okada et al., 1993). Despite the considerable advances that have been made in our understanding of the cellular mechanisms of respiratory rhythmogenesis in mammals, the use of *in vivo* models and a variety of *in vitro* models of different types, and of mammals of different developmental ages, makes comparisons among studies difficult.

In vitro brainstem models from amphibians offer some advantages over mammalian models (Luksch et al., 1996), particularly with respect to developmental regulation of the brainstem respiratory network. These advantages include a lower tissue metabolic rate that allows brainstem preparations to be maintained at their normal temperature, preventing tissue hypoxia. For example, P_{O_2} measurements within the core of the *in vitro* tadpole brainstem have been shown to be normoxic to slightly hyperoxic (Torgerson et al., 1997a). In addition, changes in central chemosensitivity during development have been measured in bullfrog tadpoles, indicating the viability of the preparation over a large range of developmental stages (Torgerson et al., 1997b, 2001a). A unique advantage of the present study compared with similar studies in mammals is that identical techniques (i.e. whole brainstem preparation *in vitro*) were used to examine respiratory rhythmogenesis in both larval and adult animals. Thus, the effects of development alone were examined in the present study without the possible confounding effects of different experimental conditions and models.

In the present study, fictive gill burst frequency in tadpole preparations was very similar to gill ventilation rates in free-swimming tadpoles (Burggren and West, 1982; Burggren and Doyle, 1986) and in spontaneously breathing, decerebrate tadpoles (Gdovin et al., 1998) at similar developmental stages. Lung burst frequency, however, was somewhat higher (0.8 min^{-1}) than the *in vivo* studies (0.1–0.3 breaths min^{-1}). One possible explanation is that MS-222 facilitates fictive lung ventilation. MS-222 is a sympathetic stimulant that increases plasma catecholamine levels for several hours in rainbow trout *Oncorhynchus mykiss* (Gingerich and Drottar, 1989) and increases heart rate in amphibians (Smith, 1974). However, the direct effects of MS-222 anesthesia on fictive breathing in the amphibian brainstem preparation have not been examined.

Role of Cl$^-$-mediated synaptic inhibition

An important finding of our study is that lung bursts in the adult bullfrog brainstem are abolished or significantly reduced in the presence of Cl$^-$-free aCSF. This is in contrast to the finding that Cl$^-$-free aCSF does not change lung burst frequency in tadpoles (this study) (see Galante et al., 1996). Because of these contrasting results, we examined the possibility that the effects of Cl$^-$ substitution were species-specific and unrelated to development. Lung burst activity was also abolished or significantly reduced in adult *R. pipiens*, suggesting that removal of Cl$^-$ has a greater effect on the respiratory CPG in adults than in tadpoles. Our studies with tadpole brainstem preparations are consistent with the results of a previous study (Galante et al., 1996) with tadpoles from similar developmental stages and at similar temperatures to those used in the present study. The data from tadpoles in the present study, together with the results of Galante et al. (1996), suggest that lung bursts in tadpoles are driven by 'pacemaker'-like neural activity that does not require Cl$^-$-dependent synaptic inhibition. However, because gill bursts were abolished during perfusion with Cl$^-$-free aCSF, conventional

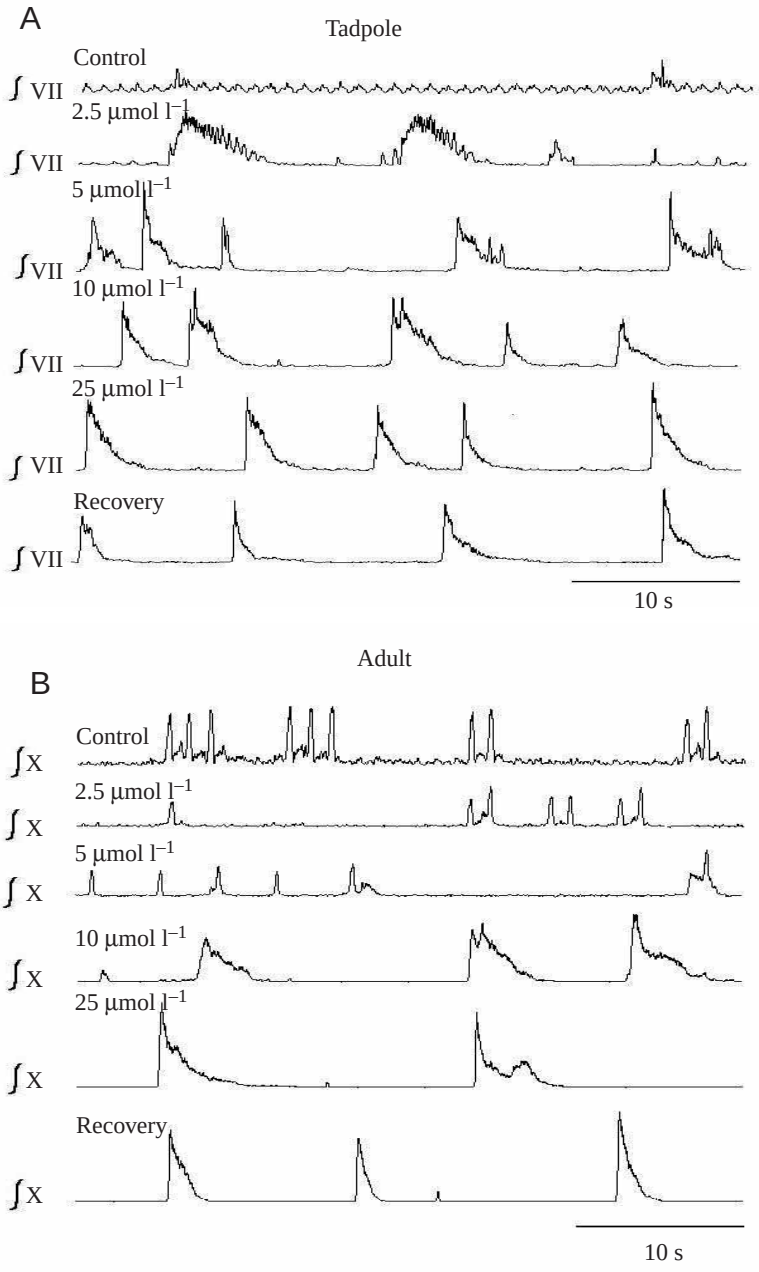

Fig. 5. Effects of increasing concentrations of the glycine receptor antagonist strychnine (from 2.5 to 25 μmol l^{-1}) on integrated neural activity (∫) from (A) a tadpole (cranial nerve VII) and (B) an adult (cranial nerve X) brainstem preparation.

Cl^--dependent inhibition may be responsible for gill bursts. These results are consistent with recent studies indicating that the gill and lung CPGs in tadpoles are anatomically separable (Gdovin et al., 1999; Torgerson et al., 2001a). The results from the present study also extend previous work to show that lung bursts in adult frogs are abolished, or their frequency significantly reduced, in the presence of Cl^--free aCSF. Cl^--dependent postsynaptic inhibition is required for many network-driven CPGs (Marder and Calabrese, 1996), and lung ventilation in the adult bullfrog appears to rely for rhythmogenesis on a postsynaptic inhibitory network that is similar to that in mammals (Ramirez et al., 1997).

The data from tadpoles and adult frogs are similar to those from studies in mammals in showing postnatal changes in the mechanism of respiratory rhythmogenesis. Superfusion of whole brainstem preparations or rhythmically active brainstem slices from neonatal rats with Cl^--free aCSF does not abolish fictive lung bursts (Feldman and Smith, 1989) or respiratory-related hypoglossal bursts (Shao and Feldman, 1997). However, perfusion of the adult *in situ* rat brainstem with a low-[Cl^-] solution reversibly abolishes respiratory activity (Hayashi and Lipski, 1992), which is consistent with the hypothesis that brainstem network interactions are responsible for driving lung ventilation in adult mammals. Although removal of extracellular Cl^- does not disrupt respiratory rhythm in the tadpole and neonatal rat brainstem, there are significant effects on lung burst properties in both preparations. In tadpoles, there was a nearly fourfold increase in burst amplitude in the presence of Cl^--free aCSF. This is consistent with a similar study with an *in vitro* tadpole brainstem preparation (Galante et al., 1996) and with increased burst amplitude in the neonatal rat brainstem (Feldman and Smith, 1989). These results suggest that Cl^--mediated inhibition is not important for rhythm generation but may affect neurons involved in pattern formation. It has been suggested that the respiratory rhythm and pattern are generated by separate mechanisms in mammals (Feldman and Smith 1989; Smith et al., 2000).

Removal of extracellular Cl^- would be expected to cause depolarization of respiratory neurons as a result of the diffusional efflux of intracellular Cl^-, and this change in membrane potential (E_m) might have different effects on the respiratory CPG in tadpoles and adults. Changes in E_m are important for modulating respiratory-related pacemaker cell frequency within the pre-Bötzinger complex in brainstem slices from neonatal rats (Smith et al., 2000). Thus, removal of extracellular Cl^- might induce changes in tonic excitation that could drive the cell voltage out of the range for expressing respiratory activity in our amphibian preparations. We do not believe this is the case because varying tonic excitation within the entire brainstem by changing extracellular [K^+] results in significant, linear changes in fictive gill/lung ventilation in tadpoles (R. E. Wade and M. S. Hedrick, unpublished results). Therefore, it seems unlikely that changes in tonic excitation induced by Cl^- efflux in these experiments would

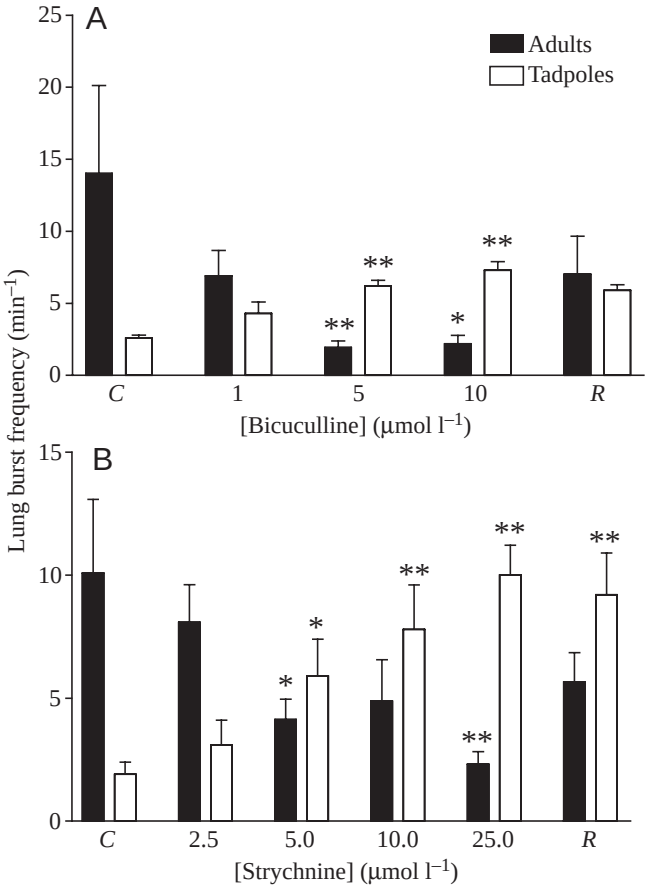

Fig. 6. Summary of the effects of (A) bicuculline and (B) strychnine on fictive lung burst frequency (min^{-1}) for tadpoles (N=7) and adults (N=7). Values are means + S.E.M. *P<0.05, **P<0.01 compared with the control value. *C*, control value; *R*, recovery value.

completely explain the different responses of tadpoles and adults to Cl^--free aCSF.

In the present study, both GABA and glycine produced an overall inhibition of respiratory activity in tadpoles and in adults. The inhibitory effects of GABA and glycine are consistent with the study by Galante et al. (1996) and with the effects of microinjections of GABA into rostral areas of the adult bullfrog brainstem (McLean et al., 1995). Adult bullfrogs showed a significant reduction in lung burst frequency at an approximately 10-fold lower concentration of GABA or glycine compared with tadpoles, suggesting a greater sensitivity to these transmitters in the mature brainstem. This may imply an increased density of GABA and/or glycine receptors in adults compared with tadpoles or differences in synaptic uptake mechanisms, but the development of GABA and/or glycine receptors in the amphibian brainstem has not been investigated. Differences in diffusion of the agonists to respiratory neurons might explain these results, but this is unlikely. Because the brainstem of adults is slightly larger than that of tadpoles, diffusion distances will probably be greater, and we would expect a lower sensitivity to GABA and glycine if diffusion alone accounted for these results.

There were also differences in the response characteristics to GABA in tadpoles and adults. In tadpoles, GABA produced a biphasic response, with excitation of lung burst frequency at lower concentrations followed by inhibition at higher concentrations, whereas GABA produced only inhibition of lung ventilation in adults. In various regions of the mammalian brain, GABA and glycine are depolarizing during earlier developmental stages (Cherubini et al., 1991), but become hyperpolarizing during maturation (Garaschuk et al., 1998). There is recent evidence for simultaneous excitation and inhibition by GABA in the developing hippocampus (Ben-Ari, 2001). The transition from excitation to inhibition is due to a change in the accumulation of intracellular Cl^- and a shift in the equilibrium potential for Cl^- (Grillner, 1999). Developmental differences in cellular Cl^- homeostasis might explain the excitatory effects of GABA on tadpoles, but this possibility has not been examined in amphibians.

The results of the present study are also similar to those of studies with mammals in which GABA or glycine receptor blockade augments respiratory-related burst frequency and amplitude in rhythmically active brainstem slices from neonates (Shao and Feldman, 1997), but abolishes or reduces respiratory-related activity in adults (Hayashi and Lipski, 1992; Ramirez et al., 1996; Pierrefiche et al., 1998). In maturing mice, lung burst frequency and pattern becomes increasingly sensitive to the effects of strychnine during postnatal life (Paton and Richter, 1995; Ramirez et al., 1996). However, GABA and/or glycine receptor blockade fails to abolish respiratory-related activity in neonatal rat brainstem preparations (Feldman and Smith, 1989), rhythmically active brainstem slices (Shao and Feldman, 1997) or working heart/brainstem preparations from adult mice (Büsselberg et al., 2001). Bilateral injections of strychnine and bicuculline into the pre-Bötzinger complex of adult cats *in vivo* abolishes respiratory motor activity, illustrating the importance of synaptic inhibition for respiratory rhythm generation in adult mammals (Pierrefiche et al., 1998). Perfusion of whole rat brainstem with bicuculline and/or strychnine disrupts normal respiratory rhythm, but breathing is not abolished (Hayashi and Lipski, 1992).

The lung burst frequency of tadpoles increased in response to bicuculline or strychnine, whereas similar concentrations of these drugs decreased lung burst frequency in adult bullfrog preparations (Fig. 6). Application of the $GABA_B$ antagonist 2-OH-saclofen produces dose-dependent increases in lung burst frequency in pre-metamorphic (Taylor–Köllros stages VI–X) tadpoles, but inhibits lung bursts in late-stage (Taylor–Köllros stage 24), post-metamorphic tadpoles (Strauss et al., 2000). Although the tadpoles in the present study were from intermediate stages, the similarity between our results and those of Strauss et al. (2000) strongly suggests that GABAergic, and also glycinergic, inputs regulate the respiratory CPG during development.

Our results indicate that, during development in *Rana catesbeiana*, lung ventilation is primarily pacemaker-driven, but becomes increasingly dependent upon fast Cl^- synaptic

inhibition mediated by GABA and glycine receptors. A shift from a pacemaker-driven to a network-dominated respiratory CPG has also been hypothesized to occur during development in mammals (Smith et al., 2000). Thus, there may be common developmental changes in the neural processes underlying lung ventilation in vertebrates. Our results suggest that developmental maturation of the bullfrog respiratory CPG may reflect developmental changes in glycinergic and/or GABAergic synaptic inhibition.

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