Role of chloride-mediated inhibition in respiratory rhythmogenesis in an *in vitro* brainstem of tadpole, *Rana catesbeiana*

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1. The isolated brainstem of larval *Rana catesbeiana* maintained *in vitro* generates neural bursts that correspond to the lung and gill ventilatory activity generated in the intact specimen. To investigate the role of chloride channel-dependent inhibitory mechanisms mediated by $GABA_A$ and/or glycine receptors on fictive lung and gill ventilation, we superfused the isolated brainstems with agonists, antagonists (bicuculline and/or strychnine) or a chloride-free solution while recording multi-unit activity from the facial motor nucleus.

2. Superfusion with the agonists (GABA or glycine) produced differential effects on frequency, amplitude and duration of the neural bursts related to lung and gill ventilation. At a GABA or glycine concentration of 1·0 mM, fictive gill bursts were abolished while fictive lung bursts persisted, albeit with reduced amplitude and frequency.

3. At the lowest concentrations used (1·0–2·5 μM), the $GABA_A$ receptor antagonist bicuculline produced an increase in the frequency of lung bursts. At higher concentrations (5·0–20 μM) bicuculline produced non-specific excitatory effects. The glycine antagonist strychnine, at concentrations lower than 5·0 μM, caused a progressive decrease in the frequency and amplitude of the gill bursts and eventually abolished the rhythmic activity. At higher concentrations (7·5 μM), non-specific excitatory effects occurred. Superfusion with bicuculline (10 μM) and strychnine (5 μM) combined abolished the neural output for gill ventilation but increased the frequency, amplitude and duration of lung bursts.

4. Superfusion with Cl^--free solution also abolished the rhythmic neural bursts associated with gill ventilation, while it significantly increased the amplitude (228 ± 51%; $P < 0.05$) (mean ± S.E.M.) and duration of the lung bursts (3·5 ± 0·1 to 35·3 ± 3·7 s; $P < 0.05$) and improved the regularity of their occurrence.

5. We conclude that different neural systems generate rhythmic activity for lung and gill ventilation. Chloride-mediated inhibition may be essential for generation of neural bursts associated with gill ventilation. In contrast, the burst associated with lung ventilation can be generated in the absence of Cl^--mediated inhibition although the latter plays a role in shaping the normal lung burst.

Over the last two decades, investigations of the neural mechanisms responsible for respiratory rhythm and pattern generation have contributed extensively to our understanding of the control of ventilation and respiratory rhythmogenesis. To explain the origin of respiratory rhythmicity, both a network-driven oscillator and neurons having intrinsic pacemaker properties have been proposed. The network-driven oscillatory systems rely on inhibitory interactions within the network to generate the rhythm; these are likely to be mediated by inhibitory amino acids and opening of chloride channels (Ballantyne & Richter, 1984; Richter, Ballantyne & Remmers, 1986; Haji, Takeda & Remmers, 1992; Ogilvie, Gottschalk, Anders, Richter & Pack, 1992). In contrast, pacemaker theories propose that

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respiratory oscillations originate in a group of neurons inherently capable of generating a rhythm (Feldman & Smith, 1989). A pacemaker-driven respiratory oscillation has been observed in a preparation of a neonatal rat brainstem *in vitro* (Feldman & Smith, 1989; Onimaru, Arata & Homma, 1989). In addition, there may be a system that includes pacemaker cells embedded within a network that produces a rhythmic motor output. Such a hybrid system has been proposed as the basis for respiratory rhythm generation in mammals (Feldman *et al.* 1990).

The neural mechanisms of respiratory rhythm generation have been investigated almost exclusively in mammals (see Ezure, 1990, for references). However, much of our fundamental knowledge about pattern generators comes from studies in non-mammalian species, e.g. lamprey spinal cord (Vinay & Grillner, 1992), the stomatogastric ganglia of the lobster (Miller & Selverston, 1982). Such studies of the neural basis of respiration in non-mammalian species such as aplysia (Byrne & Koester, 1978), mollusc (Syed, Bulloch & Lukowiak, 1990), lamprey (Rovainen, 1977) and frog (Kogo, Perry & Remmers, 1994; Kogo & Remmers, 1994) are, however, somewhat limited.

As outlined in the accompanying manuscript (Liao, Kubin, Galante, Fishman & Pack, 1996), amphibia are an attractive species in which to study the neural basis of respiratory rhythm and its development. However, the act of breathing in amphibia is different from mammals. They use a buccal force pump mechanism (deJongh & Gans, 1969), by which ambient air is forced into the lungs by pressure generated by the buccal musculature. A similar mechanism is employed by the African lungfish (McMahon, 1969), which is close to amphibia in the evolutionary tree. In the lungfish, as in mammals, vagal afferent information from the lung has a similar effect on the control of respiratory timing (see Pack, Galante, Walker, Kubin & Fishman, 1993). In keeping with the observations in lungfish and mammals, vagotomy in frogs prolongs the duration of lung inflation (Evans & Shelton, 1984). These observations suggest that many components of the respiratory timing mechanism, typical of mammals, exist in animals who rely on a buccal force pump for breathing. Thus, a study of the neural mechanisms underlying respiratory rhythm generation in amphibia could provide important insights into the evolution of respiratory control in mammals.

The goal of this study was to evaluate the role of chloride-mediated inhibition in generating the respiratory rhythm for both lung and gill ventilation in an anuran larvae (tadpole). In order to study respiratory rhythm generation in the brainstem of the tadpole we utilized an isolated *in vitro* brainstem preparation developed for this and the accompanying manuscript (Liao *et al.* 1996). Larval *Rana catesbeiana*, at intermediate stages of development, were used because they have both lung and gill ventilatory output (Burggren & West, 1982). Since both of these ventilatory acts involve cranial motoneurons, we recorded activity from the facial motor nucleus to monitor fictive ventilatory output. In the first series of experiments, we tested the role of chloride-mediated inhibition on the generation of lung and gill rhythms by superfusing the tadpole brainstem with progressively increasing concentrations of two inhibitory amino acids: GABA and glycine. Subsequently, to assess the role of endogenous inhibition we superfused the preparation with inhibitory amino acid receptor antagonists. Finally, we addressed the question of whether either of the rhythms would persist if chloride-mediated inhibition were abolished either by removal of Cl^- from the medium or using a combined superfusion with antagonists of both $GABA_A$ and glycine receptors.

Preliminary reports have been communicated (Pack, Galante, Walker, Kubin & Fishman, 1991; Galante, Smith, Kubin & Pack, 1992; Pack *et al.* 1993).

METHODS

Experimental animals

Successful studies were carried out in thirty-five larval bullfrogs (tadpoles), *Rana catesbeiana*, at developmental stages XVI–XIX (9·1 ± 3·4 g body weight). The metamorphosis from tadpole to adolescent frog is based on gross morphological changes (for full details see Taylor & Kollros, 1946). At the stages of development in the studies reported here, tadpoles have functional lungs and gills (Burggren & West, 1982). All tadpoles were obtained from a commercial supplier (Nasco, Fort Atkinson, WI, USA). They were maintained in filtered water at 20 ± 2 °C and fed granulated Purina Trout Chow (Ralston Purina Co., St Louis, MO, USA) *ad libitum* for at least 5 days before the experiments were done.

Surgical preparation, perfusion and recording chamber

The tadpoles used in these studies were anaesthetized by using the techniques described in the accompanying manuscript (Liao *et al.* 1996). We also used the surgical techniques described therein, the same perfusion system, superfusate and recording chamber. The concentration of CO_2 bubbled in the superfusate was adjusted to maintain the pH in the bath at 7·4. At this stage of development at a P_{CO_2} of 5 Torr, a typical brain extracellular pH for the larval form of *Rana catesbeiana* would be 7·8 (Just, Gatz & Crawford, 1973). However, we chose a pH of 7·4 since this more acidic pH increases lung ventilation but not buccal (gill) ventilation (Kinkead, Filmyer, Mitchell & Milsom, 1994). Under these conditions, the isolated brainstem tissue was viable for at least 6 h, as judged by the stability of neural recordings.

Recording techniques

Multi-unit activity was recorded extracellularly from the VII motor nucleus using tungsten microelectrodes (tip diameter, 100–200 μm) with a resistance of 500–700 kΩ. We did not, in this study, attempt to record from single motoneurons. Electrodes were inserted through the dorsal surface of the medulla on the left or right side using a manually driven micromanipulator (MM3, Stoelting, Chicago, IL, USA). Co-ordinates of the recording sites were determined using the obex as a reference. Typical recording positions were located 2·5–2·7 mm rostral to the obex and

0·3–0·5 mm off the mid-line. Extracellular activity was fed to a high impedance preamplifier (HSA 830, CWE Inc., Ardmore, PA, USA), and amplified further, then filtered (1 Hz to 10 kHz) using another AC amplifier (BMA 830, CWE). A moving average of the raw signal was obtained by passing it through a full-wave rectifier circuit and a third-order Paynter filter (MA821, CWE) having a time constant of 50–200 ms (typically 100 ms). The outputs of the amplifier and moving averager circuit were fed to a potentiometric recorder (ES-1000, Gould) and stored on an FM tape recorder (3960, Hewlett-Packard) for subsequent data analysis.

Drugs

GABA, glycine, bicuculline methiodide and strychnine were obtained from Sigma. The solutions were freshly prepared before each experiment using the control superfusion medium as the vehicle.

Experimental protocols

We administered two inhibitory agonists, GABA and glycine, and two antagonists, bicuculline ($GABA_A$ receptors) and strychnine (glycine receptors). In addition, because both types of receptors are associated with membrane channels for Cl^-, the effect of replacing Cl^- in the superfusate with gluconate was examined. Except for the experiments in which both bicuculline and strychnine were administered, only one compound was studied in each experiment. All studies had a control period, a period of drug administration at increasing concentrations and, whenever possible, a post-drug control period.

Control. For each experiment, control recording began at least 60 min after surgery was completed. Then, from the VII motor nucleus we determined the frequency and amplitude of the gill, and lung bursts and the duration of the lung burst during a 30 min period. Steady-state measurements for gill breathing were made between lung breaths taken 15–30 s after the end of a lung breath throughout the period until at least 15–30 s before the onset of the next lung breath.

Application of inhibitory amino acid receptor agonists and antagonists. In preliminary studies we determined that the concentration of glycine and GABA that produced minimal to maximal effects on neural output for gill and lung ventilation were 0·1–5·0 mM. Similarly, the appropriate concentrations for bicuculline were 1·0–20·0 μM and for strychnine 0·25–10·0 μM.

During the test period, the drugs were applied by superfusion using alternately two identical perfusion systems. Agonists or antagonists were superfused with progressively increasing concentrations by switching between the two systems. At each concentration, steady-state conditions were established by allowing 15 min for agonists and 3–5 min for antagonists. After a trial with the maximal concentration of each compound, the brainstem was again superfused with the control solution in an attempt to return to control conditions; at least 15–30 min of washout were allowed for agonists and 60–90 min for antagonists. In some experiments involving antagonists, we were unable to obtain recordings fully comparable to those during the initial control period even after 90 min of superfusion with the control solution. In total, six complete experiments were done with glycine, six with GABA, six with bicuculline and six with strychnine.

In a separate series of experiments in four tadpoles, we studied the combined effects of strychnine (5 μM) and bicuculline (10 μM). This protocol consisted of a 30 min control, 10–15 min exposure to the drugs, and a 60–90 min washout to re-establish control conditions.

Chloride ion replacement. In seven tadpoles, we used a solution in which gluconate replaced Cl^- in the superfusate. The composition of the Cl^--free solution was (mM): sodium gluconate, 78·8; potassium gluconate, 3·1; calcium gluconate, 3·2; $MgSO_4$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 30; glucose, 3·9 (pH 7·4, 20 °C). Since gluconate acts as a calcium chelator (Christoffersen & Skibsted, 1975), 3·2 mM calcium gluconate had to be added to the solution to achieve the same concentration of ionized calcium as in control conditions, as measured using a calcium electrode (Stat Profile 5, Nova).

Following a 30 min control period, we switched to the Cl^--free superfusate. Fifteen minutes were allowed to establish steady-state conditions. This time interval was chosen since, in our preliminary studies, we found that a steady-state rhythm was generated after this period. We then determined the effects of Cl^--free solution over a 15 min period, following which we switched again to the control solution. A period of 60–90 min was allowed for the neural output to return, if possible, to the control pattern.

Statistical analysis

The effects of GABA, glycine and Cl^- removal on lung and gill burst frequency, amplitude and duration were analysed statistically using a one-factor analysis of variance (ANOVA) with repeated measures. The criterion for statistical significance was $P < 0·05$. All values are given as the mean ± S.E.M.

The statistical significance of the differential effect of GABA or glycine on gill and lung bursting activity was examined using within-animal comparisons. For each concentration of GABA or glycine in the superfusate, the lung burst frequency, as a percentage of the control value, was subtracted from the gill burst frequency, as a percentage of the control value. The null hypothesis for these tests was that it is equally possible for gill frequency and lung frequency to undergo the larger decline in individual animals. After confirming that the distributions of differences were sufficiently normal to permit parametric analyses, Student's paired t tests were used with or without a Bonferroni adjustment depending on the number of comparisons performed. An identical analysis for the amplitude of the lung and gill bursts in these experiments was performed.

Non-parametric comparisons were used in the analyses of gill and lung ventilation in experiments in which Cl^- was removed from the superfusate when the relevant distributions did not appear to be normal.

RESULTS

In these studies we recorded, as reported in the companion manuscript (Liao *et al.* 1996), two major types of burst from the vicinity of the VII motor nucleus (see Fig. 1). There was a relatively fast lower amplitude burst that we believe is that related to gill ventilation, i.e. gill bursts. The mean frequency of these bursts was 33·6 ± 1·1 min^{-1} averaged across all animals studied. The other burst was slower and had a higher amplitude (see Fig. 1), and is related to that for lung ventilation, i.e. lung bursts. The mean frequency of these bursts, averaged across all animals studied, was 40·7 ± 10·2 h^{-1}.

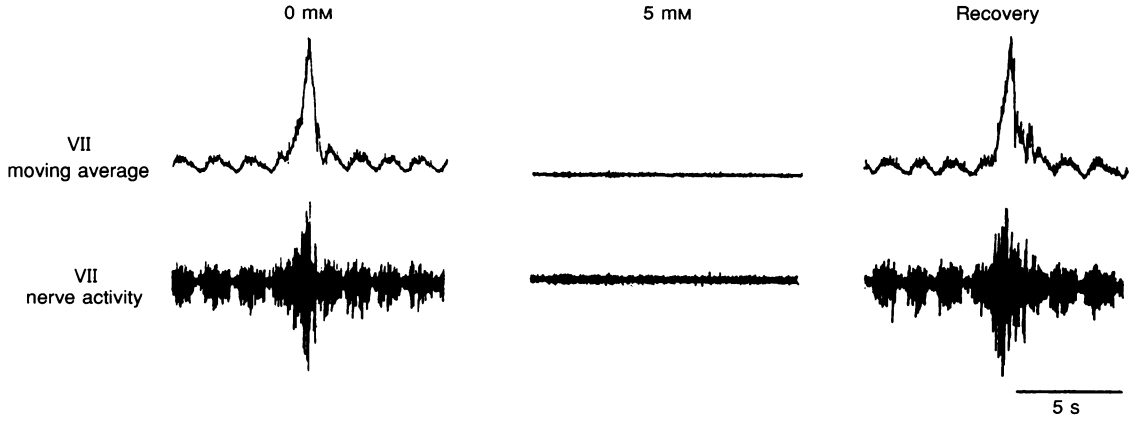

Figure 1. Recordings of multi-unit activity from VII motor nucleus and the corresponding moving average from one isolated brainstem preparation in control, after superfusion of 5 mM of GABA, and recovery

There are two types of bursts seen in both the control (left panel) and recovery (right panel) recordings: a low amplitude, more frequent gill burst and a higher amplitude less frequent, lung burst. At a GABA concentration of 5 mM, lung and gill bursting activity is abolished.

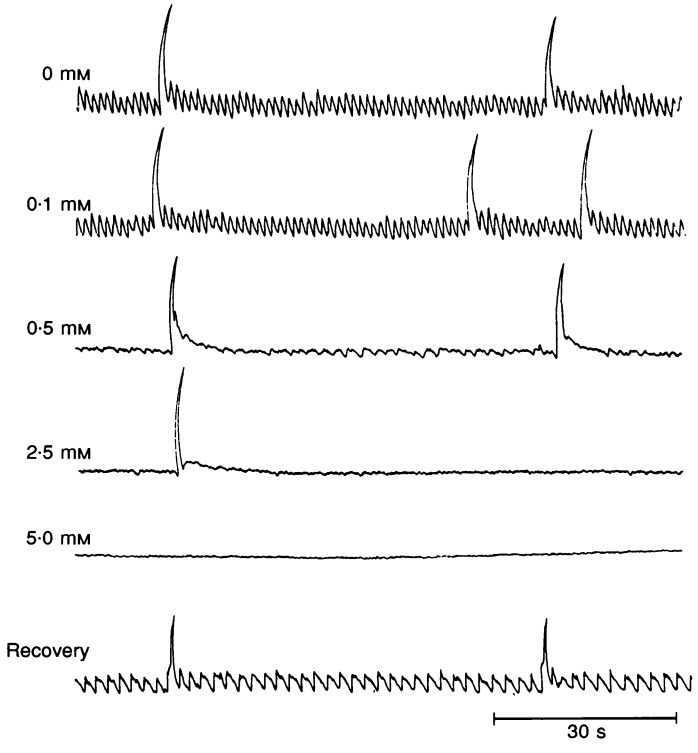

Figure 2. Effects of GABA on neural activity related to fictive lung and gill ventilation in the *in vitro* tadpole brainstem preparation

The records show moving averages of multi-unit neural activity recorded from the VII motor nucleus at different concentrations of GABA in the medium. The fictive gill ventilation (small amplitude, fast oscillations seen in the two top and the bottom records) is abolished at lower concentrations than the bursts for fictive lung ventilation (larger amplitude, slower frequency). At the highest concentrations of GABA (5·0 mM) both rhythms were abolished. In this and all subsequent figures, all recordings were made at the same amplifier gain and without adjustment of the zero setting.

Studies with GABA

GABA, at a concentration of 5 mM, abolished all activity related to both gill and lung ventilatory bursts. This is shown in Fig. 1 where original multi-unit recordings in control, at 5 mM GABA, and following recovery are shown. The application of GABA affected the neural output, however, for gill ventilation at lower concentrations than for fictive lung ventilation. The moving average of VII motor nucleus activity from one experiment in which GABA was applied in increasing concentrations is shown in Fig. 2. The average effects of GABA at different concentrations on the burst frequencies (*A*), amplitudes (*B*) for both rhythms and duration of the lung burst (*C*) in six experiments are shown in Fig. 3. The amplitudes shown here, and throughout the results, are the amplitudes of the moving average of the multi-unit activity in the VII motor nucleus associated with each type of burst and measured from the baseline established from the segments of records where activity was absent.

At the lowest concentration of GABA (0·1 mM) we found no significant changes in any parameter related to fictive lung bursts. In contrast, gill frequency and amplitude decreased significantly ($P < 0·05$). At 0·5 mM of GABA, gill burst amplitude decreased further, to $18 \pm 12\%$ of control ($P < 0·05$), and gill frequency decreased significantly from 26 ± 4 to $6·3 \pm 5·2$ min^{-1} ($P < 0·05$), without significant effects on fictive lung bursts. This differential effect of 0·5 mM GABA on the neural activity for lung and gill ventilation occurred in all six experiments. Thereafter, with increasing concentrations of GABA, the fictive lung ventilatory frequency, amplitude and burst duration also decreased. At GABA concentrations of 1·0 and 2·5 mM, lung bursts still persisted while neural activity for gill ventilation was abolished. Abolition of all discharge associated with lung bursts consistently occurred at the highest concentration of GABA used (5 mM). Both lung and gill rhythmic activities returned within 15–30 min after washout in all six preparations studied.

Table 1 summarizes the differential nature of the effect of GABA on gill and lung frequency and amplitude. The *P* values shown are without Bonferroni correction. At 0·5 and 1·0 mM, the percentage decline of the frequency of gill bursts was significantly larger than that observed for the lung bursts, even after adjustment for multiple comparisons.

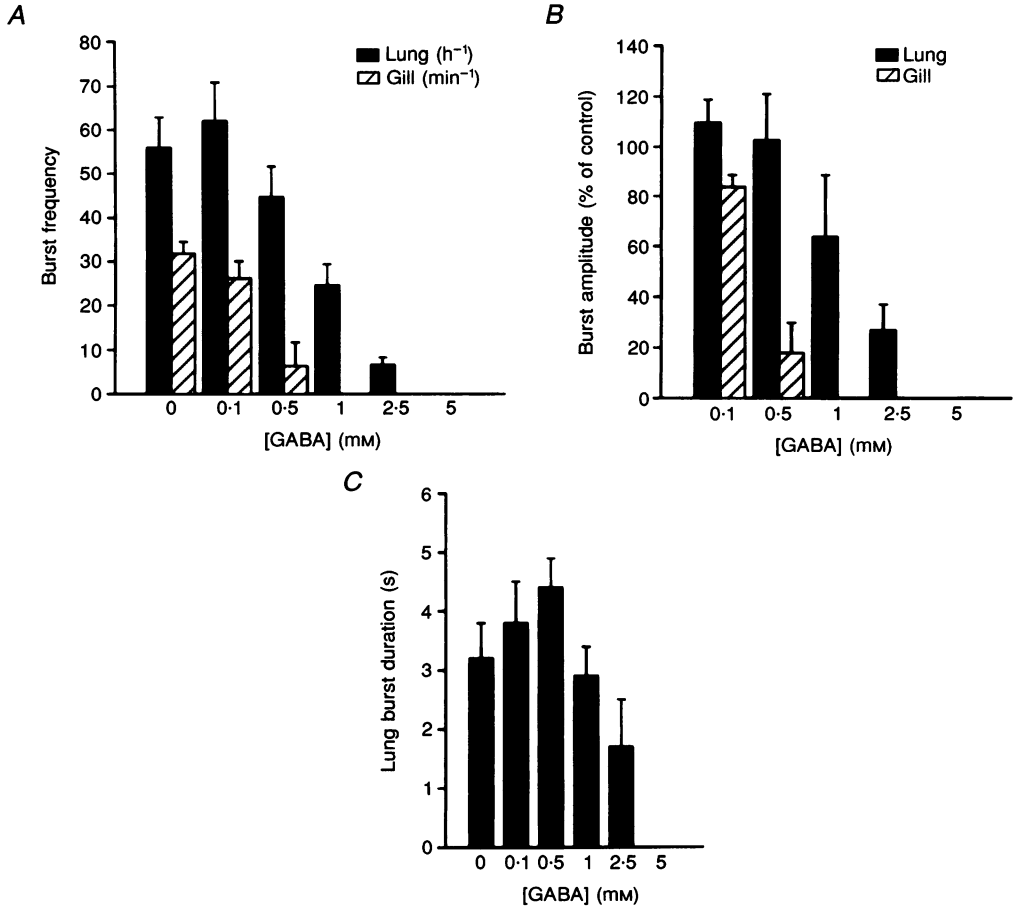

Figure 3. Average effects of increasing concentrations of GABA on lung and gill burst frequency (*A*), lung and gill burst amplitude (*B*) and lung burst duration (*C*)

Data are shown as mean ± S.E.M. for six experiments.

Table 1. Differential effects of GABA on gill and lung ventilatory frequency and amplitude

	Frequency			Amplitude		
Concentration (mM)	Gill (%)	Lung (%)	*P* value*	Gill (%)	Lung (%)	*P* value*
0·1	−17·2	+11·4	0·06	−16·4	+9·7	0·03
0·5	−83·4	−20·5	0·007	−82·0	+2·6	0·02
1·0	−100·0	−51·4	0·008	−100·0	−36·5	0·05
2·5	−100·0	−86·1	0·02	−100·0	−73·1	0·04
5·0	−100·0	−100·0	n.a.	−100·0	−100·0	n.a.

Values are the mean relative change (%). * *P* values for differences in lung and gill change. n.a., not applicable.

For the amplitude of the gill burst, the percentage decline was significantly larger than that for lungs at concentrations of 0·1, 0·5, 1·0 and 2·5 mM, although none of the changes were significant after adjustment for multiple comparisons.

Studies with glycine

Glycine in increasing concentrations (0·05–5 mM) was applied by superfusion to six tadpole brainstems. As with GABA, glycine caused a concentration-dependent suppression of activity with differential effects on the fictive lung and gill motor output in all six experiments. Figure 4 shows recordings obtained from one animal; Fig. 5 shows the average data. Significant changes in lung burst duration, amplitude and frequency did not occur until the concentration of glycine was 1 mM. At this concentration the average frequency of lung bursts decreased from a control value of $52{\cdot}7 \pm 14{\cdot}9$ to $12 \pm 5{\cdot}9\ h^{-1}$ ($P < 0{\cdot}05$), the duration of the burst decreased from $2{\cdot}7 \pm 0{\cdot}5$ (control) to $1{\cdot}8 \pm 0{\cdot}2$ s ($P < 0{\cdot}05$), while the amplitude fell to $28{\cdot}6 \pm 9{\cdot}3\%$ ($P < 0{\cdot}05$) of control. There was a further significant decrease in lung burst amplitude, duration and frequency at a glycine concentration of 2·5 mM, and an abolition of lung bursting occurred in all tadpoles at 5 mM.

The concentration dependence of glycine effects on gill bursts had a different pattern. At a concentration of 0·5 mM, the decrease in gill burst frequency became

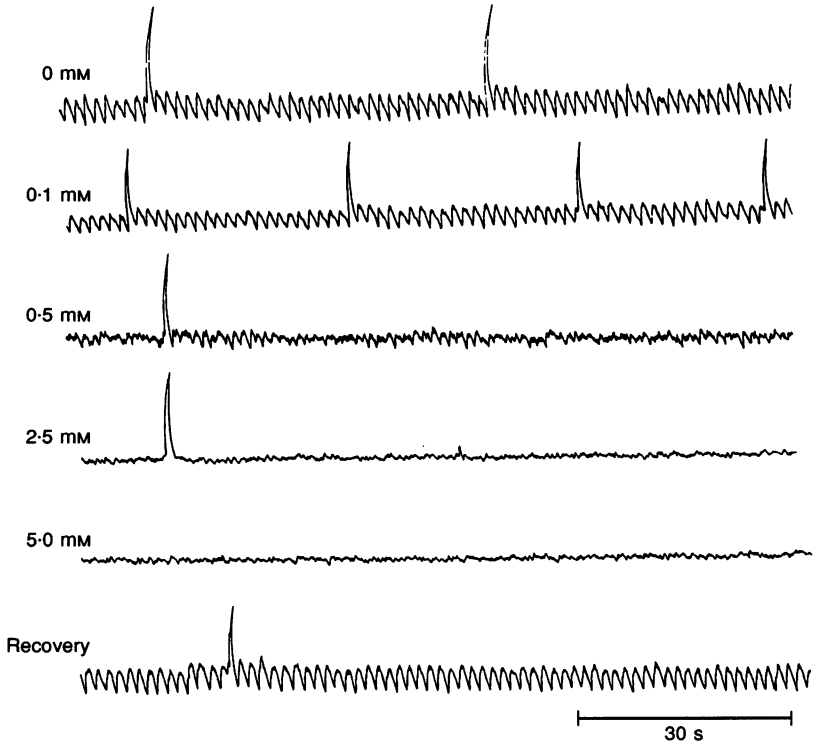

Figure 4. Effects of glycine on fictive lung and gill ventilation

There was a differential effect on lung and gill ventilation, with gill ventilation being more sensitive to the inhibitory effects of glycine. At the highest concentration (5 mM) both gill and lung bursting was abolished. See Fig. 2 for explanation of traces.

Table 2. Differential effects of glycine on gill and lung ventilatory frequency and amplitude

	Frequency			Amplitude		
Concentration (mM)	Gill (%)	Lung (%)	*P* value*	Gill (%)	Lung (%)	*P* value*
0·05	−9·1	+28·8	0·11	+7·5	+5·5	0·56
0·1	−18·9	+40·2	0·04	−11·1	−12·0	0·69
0·5	−79·7	−28·4	0·22	−41·0	−50·2	0·004
1·0	−100·0	−82·0	0·02	−100·0	−71·4	0·03
2·5	−100·0	−82·0	0·02	−100·0	−86·5	0·10
5·0	−100·0	−100·0	n.a.	−100·0	−100·0	n.a.

Values are the mean relative change (%). * *P* values for differences in lung and gill change.

significant (from $33{\cdot}1 \pm 4{\cdot}1$ min^{-1} in control to $7{\cdot}8 \pm 7{\cdot}1$ min^{-1}, $P < 0{\cdot}05$) as was a $41{\cdot}0 \pm 5{\cdot}3$% ($P < 0{\cdot}05$) decrease in amplitude. At the next higher concentration of glycine (1 mM), gill bursts were abolished while lung bursts continued in all six experiments.

Table 2 presents the analyses of the percentage changes in the frequency and changes in the amplitude of the lung and gill bursts after glycine. For the frequencies, individual differences at 0·1, 1·0 and 2·5 mM were significant, although none of the comparisons had significance levels below the conservative (Bonferroni-adjusted) level of 0·05/0·06 or 0·008. The largest difference was observed at a concentration of 0·1 mM. For burst amplitudes, a statistically significant difference in the percentage decline occurred at 0·5 mM even with the Bonferroni-adjusted *P* level of 0·008. The comparison at 1·0 mM was nominally significant with $P = 0{\cdot}03$.

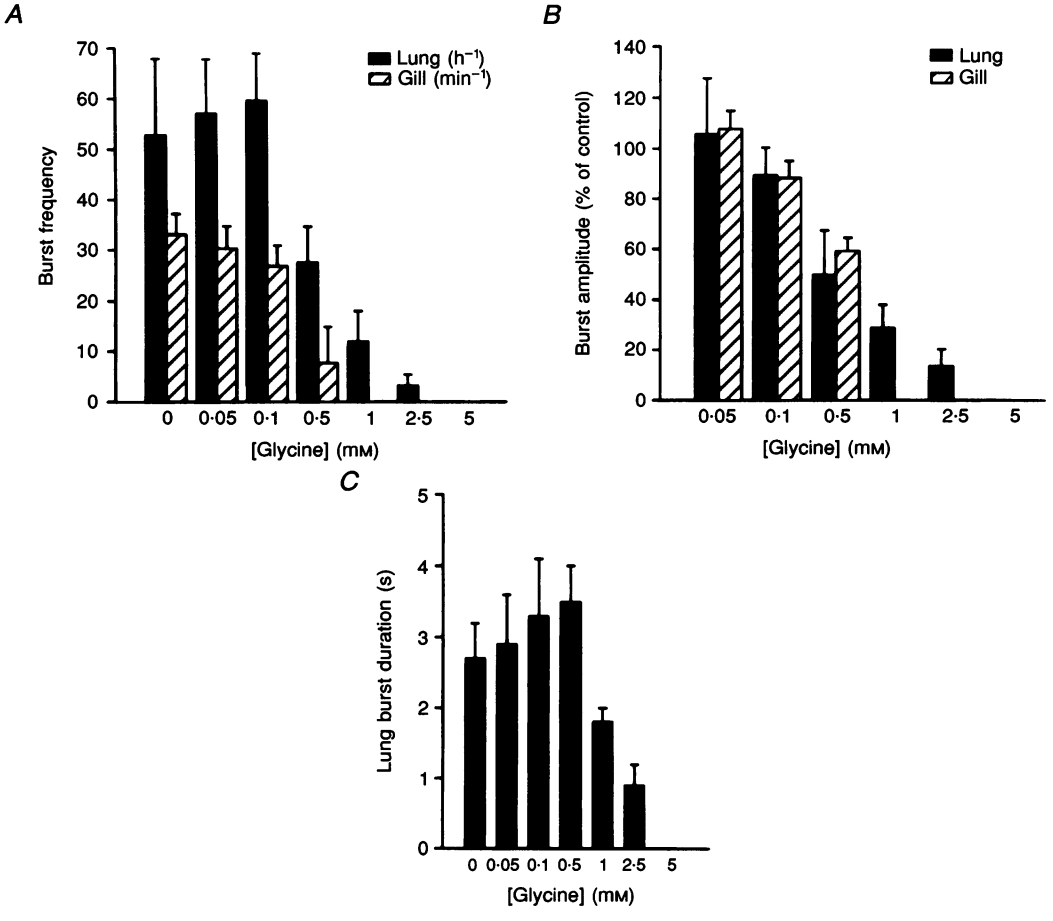

Figure 5. Average effects of increasing concentrations of glycine on lung and gill burst frequency (*A*), lung and gill burst amplitude (*B*) and lung burst duration (*C*)

Data are shown as the mean ± S.E.M. for six experiments.

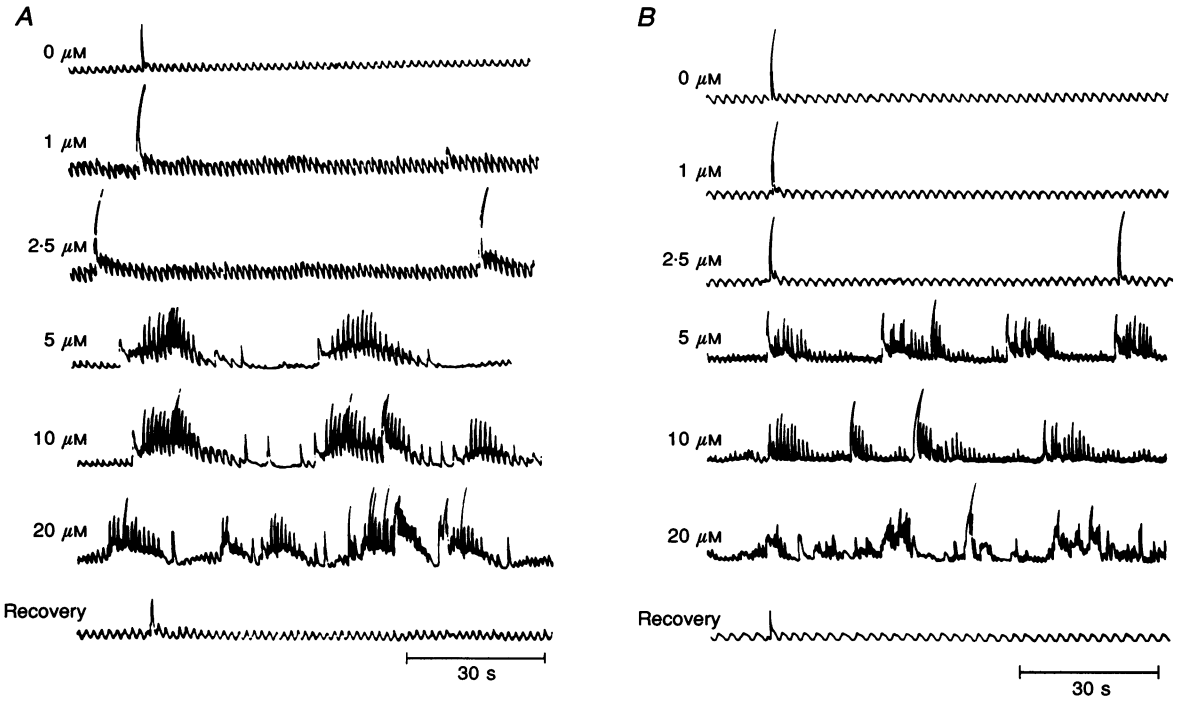

Figure 6. Effects of bicuculline

The records show lung and gill bursting activity in two experiments (*A* and *B*, respectively). At lower concentrations (1–2·5 μM) patterns typical of lung and gill activity are still present. There is an increase in lung burst frequency at these concentrations. At higher concentrations (5–20 μM) non-specific excitatory effects are observed.

Effects of antagonists, bicuculline and/or strychnine

Bicuculline. Six experiments were performed using increasing concentrations of bicuculline. The original data from two experiments are presented in Fig. 6. In all six experiments, we encountered difficulty in distinguishing bursts for gill and lung ventilation at higher concentrations of bicuculline due to non-specific excitatory effects. The non-specific effects started in the six different animals studied at 1·0, 2·5, 2·5, 2·5, 5·0 and 5·0 μM. Thus, we had data that could be analysed in three studies at a concentration of 1·0 μM and two studies at 2·5 μM. We found at 1·0 μM ($n = 3$) that the lung burst frequency

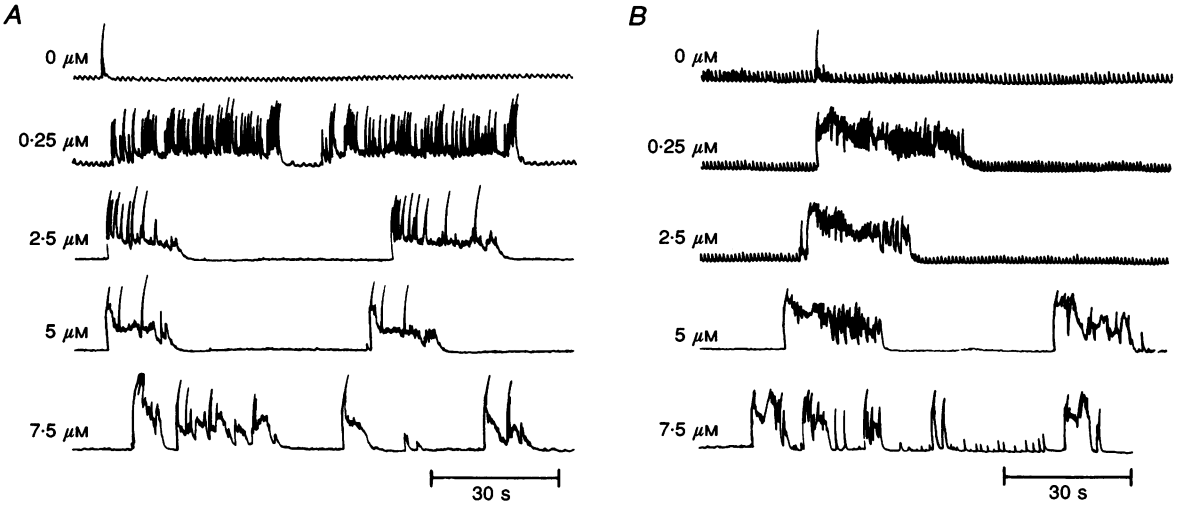

Figure 7. Effects of strychnine

The records show lung and gill bursting activity in two experiments (*A* and *B*, respectively). Gill ventilation was abolished at intermediate concentrations. A rhythmic burst continued at these concentrations but its characteristics do not allow us to conclude that it was a lung burst.

increased from a mean of 10 h^{-1} in control to 17 h^{-1} and gill burst frequency increased from 37 to 46 min^{-1}. Lung burst amplitude decreased slightly (5%) while gill amplitude increased by 35% relative to control. At 2·5 μM bicuculline ($n = 2$) there was an increase in lung frequency from a mean of 6·5 to 30 h^{-1} as well as a slight increase in gill frequency (33 to 38 min^{-1}). Lung and gill burst amplitudes were inversely affected at this concentration when compared with control: lung amplitude decreased by 12% while gill amplitude increased by 16%. Lung and gill bursts returned to control levels in four out of six experiments following 60–90 min of washout.

Strychnine. As with bicuculline, non-specific effects at higher concentrations of strychnine (5·0 and 7·5 μM) obscured individual bursts. Figure 7 shows examples of the non-specific effects of strychnine at the higher concentrations in two of the six experiments as well as the effects of strychnine at lower concentrations. At the lower concentration (0·25 μM), the gill burst amplitude and frequency in five of the six experiments were reduced while in the sixth experiment gill rhythmicity was completely abolished. In all experiments, strychnine abolished bursts for gill ventilation albeit at different concentrations, ranging from 0·25 to 5·0 μM in the six different experiments. At 0·25 and 2·5 μM, the lung bursts no longer had the same pattern of activity that was observed in control conditions. However, at these concentrations, there was a rhythmic bursting activity that could not be characterized as non-specific excitation nor could it be definitively identified as the complex bursts that were occasionally observed in control conditions (see Results, last paragraph) because of its atypical shape. This bursting pattern persisted throughout the measurement period and increased in frequency with increasing concentrations of strychnine. The mean frequency of this activity ($n = 6$), at 0·25 and 2·5 μM strychnine, increased from 24 to 40 h^{-1}, respectively. Amplitude of this activity was unaffected while the duration decreased slightly. Recovery of gill and lung ventilation was obtained in only one experiment after 60–90 min of washout.

Bicuculline and strychnine combined. To assess the combined effects of blockade of $GABA_A$ and glycine receptors, we superfused the brainstem using a solution containing 10 μM bicuculline and 5 μM strychnine in four studies. The results of this series of experiments were different from those with either of the antagonists applied alone in that the non-specific 'convulsive' effects did not occur. Examples of recordings from each of these four experiments are shown in Fig. 8. In all four experiments, gill bursts were abolished, while lung bursts persisted albeit with increased burst frequency, amplitude and duration. The bursting pattern was more complex than that in control conditions in that multiple bursts either appeared close together or seemingly as multiple oscillations within a single burst. Both lung and gill bursts returned to control levels in all four experiments after 60–90 min of washout.

Activity in the Cl^--free solution

In all seven experiments, at the beginning of superfusion with the Cl^--free solution there was a transient tonic

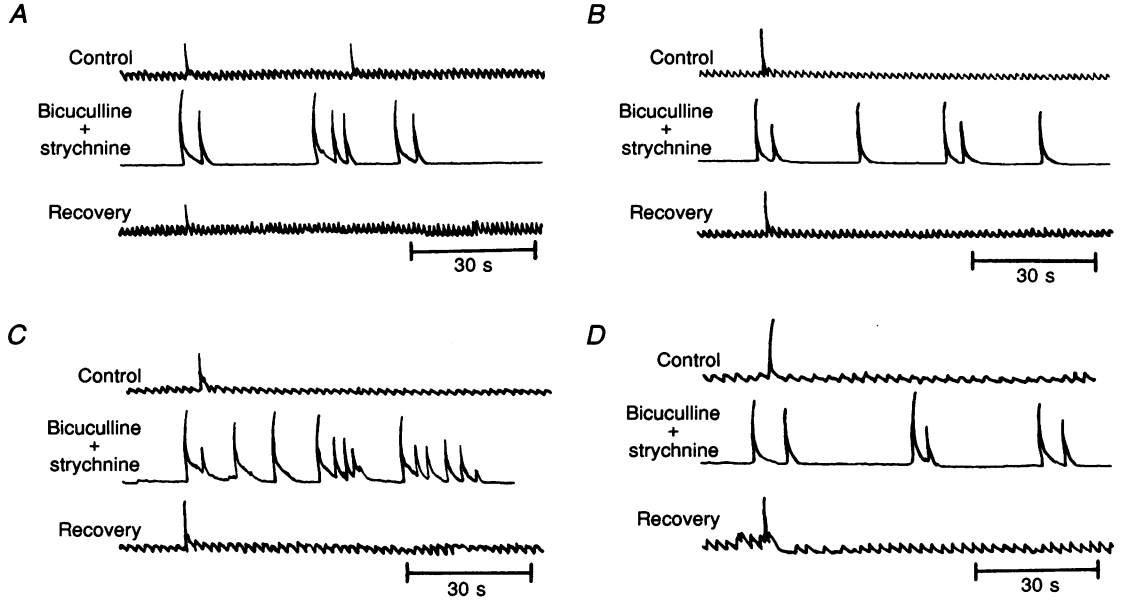

Figure 8. Effects of bicuculline and strychnine combined

The records show effects on lung and gill bursting activity in four experiments (*A–D*, respectively). Superfusion with 10 μM bicuculline and 5 μM strychnine abolished gill activity in all experiments. Lung burst frequency, amplitude, and duration increased, but the non-specific excitatory effects observed with bicuculline or strychnine alone did not occur.

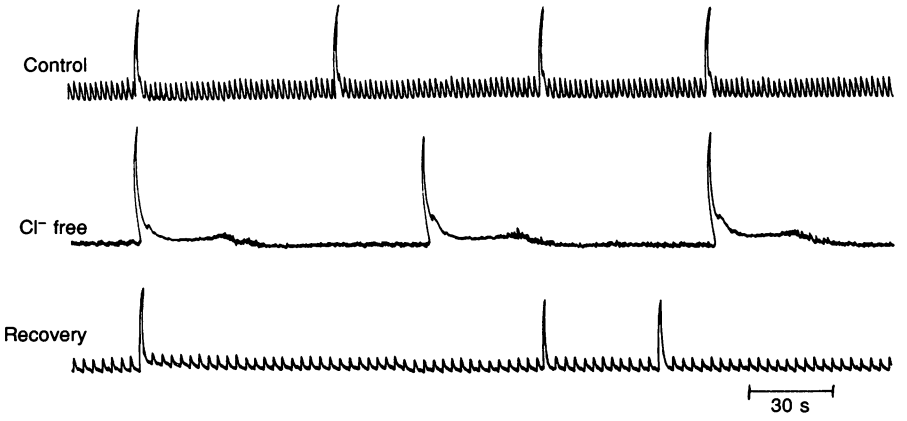

Figure 9. Example of the effect of removing Cl^- from the superfusate on lung and gill activity
In the Cl^--free solution, bursting activity related to gill ventilation was abolished while lung activity continued with increased amplitude and duration. The frequency of the lung bursts were slower and more regular under Cl^--free conditions than in control.

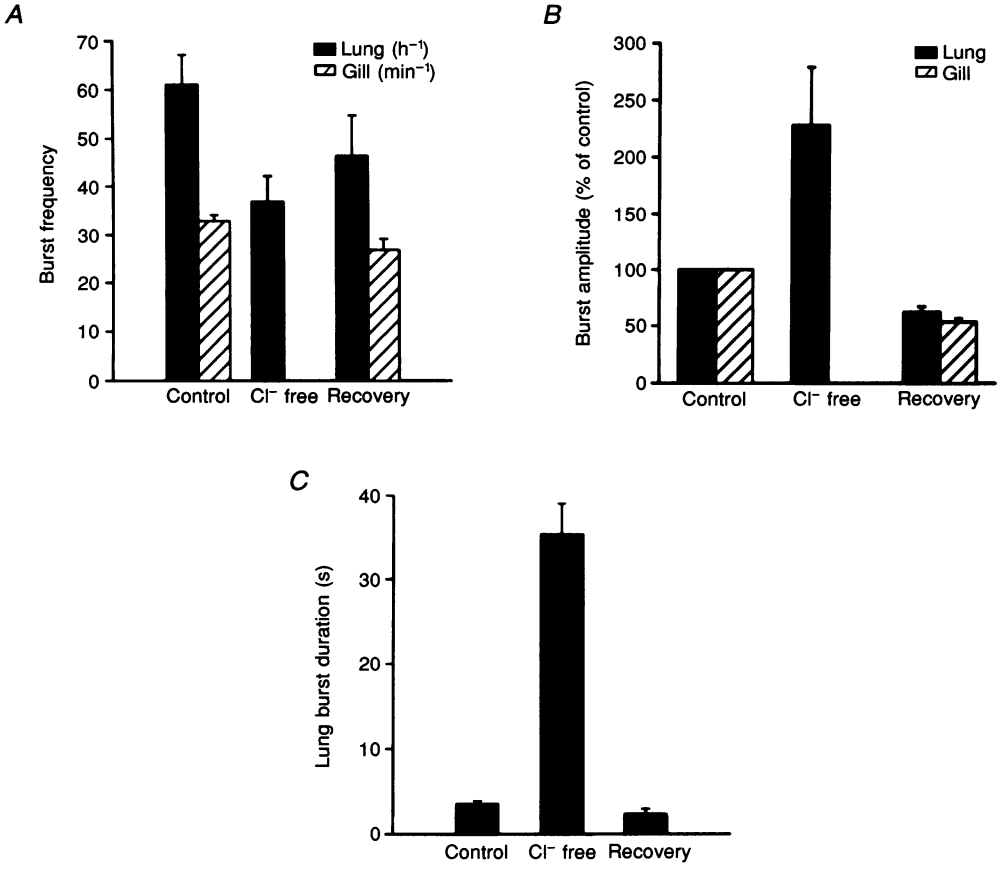

Figure 10. Average data from seven experiments showing the effects of Cl^- removal on fictive lung and gill ventilation
Under Cl^--free conditions the lung bursting activity persisted, while gill activity is abolished (*A*) and there was a significant increase in lung burst amplitude (*B*) and duration (*C*). The data for the recovery period are from 4 out of 7 experiments in which lung and gill activity returned to control levels after washout.

excitation which lasted 4–5 min. Thereafter, the rhythmic neural bursts related to gill activity ceased. In contrast, the lung bursts related to lung ventilation persisted throughout the Cl^--free recording period. Although the effect on lung burst frequency was small, burst amplitude and duration increased dramatically. An example of the response obtained in one tadpole brainstem is shown in Fig. 9, while the average data for all seven animals studied are shown in Fig. 10. The duration of neural bursts for lung ventilation increased significantly above control levels (from $3{\cdot}5 \pm 0{\cdot}1$ to $35{\cdot}3 \pm 3{\cdot}7$ s; $P < 0{\cdot}05$). The amplitude of the moving average for lung bursts also increased significantly to $228 \pm 51\%$ of control ($P < 0{\cdot}05$). The relative decrease in lung frequency was $-32 \pm 27\%$ ($P < 0{\cdot}01$). Under the Cl^--free conditions, the bursts were more regular than during the control period. An f test comparing the mean variance of the interburst interval of lung breaths in control and Cl^--free conditions demonstrated that these differences were significant ($f = 2{\cdot}10$; degrees of freedom (d.f.) = 54, 54; $P = 0{\cdot}05$). Gill bursts were abolished in all seven tadpoles during the Cl^--free period. There was, however, a low level of tonic activity between lung bursts.

In four of seven experiments, the neural bursts for both gill and lung ventilation returned after 60 min of superfusion in the control medium. In the other three preparations, the recovery was incomplete in that only gill ($n = 1$) or only lung ($n = 2$) bursts similar to those seen in control conditions were obtained.

Complex bursts

Intermittently we observed complex bursts of neural activity that differed from the high-frequency, lower amplitude bursts typical of gill ventilation or from the low-frequency, higher amplitude, stereotyped burst associated with lung ventilation. We assume that these more complex bursts might represent the lung inflation cycle that has been described in amphibia (deJongh & Gans, 1969). These complex bursts occurred in most (85%), but not all, animals studied. When present, they occurred at highly variable intervals and the duration and pattern of these complex bursts also varied within individual experiments. However, their duration was consistently longer than that associated with lung ventilation and a distinct oscillatory pattern of activity was seen within these bursts. An example of two such bursts for a single animal are given in Fig. 11. These complex bursts did not occur in Cl^--free conditions even if they were present under control conditions. Due to their irregular occurrence and variable form, these bursts were not analysed further.

DISCUSSION

The present study was undertaken to examine the role of Cl^--mediated inhibition in the generation of respiratory rhythms for both lung and gill ventilation at intermediate stages of development in the larval form of the amphibian *Rana catesbeiana*. We found that the fictive motor outputs for gill and pulmonary ventilation are differentially affected by GABA, glycine, pharmacological blockade of the inhibition produced by the inhibitory amino acids and by removal of Cl^- from the medium. Specifically, we found that agents that block Cl^--mediated inhibition abolish gill rhythmicity while maintaining lung rhythmicity. Thus, in the tadpole, fast synaptic inhibition appears to be required for generation of the gill rhythm while it is not necessary for the pulmonary rhythm.

The mechanical events associated with lung ventilation in amphibia have been extensively described (see, for example, deJongh & Gans, 1969; Sakakibara, 1984). In terrestrial anurans, including *Rana catesbeiana* (deJongh & Gans, 1969), there are two lung ventilatory acts: the lung ventilation cycle and the less frequent lung inflation cycle. This latter cycle involves a series of strokes that occur in close succession, thereby inflating the lungs to high pressures. In the present study, we observed two patterns of motor output that may be analogous to the two pulmonary ventilation modes. The first is the single lung bursts that may represent the lung ventilation cycle and the second is the intermittently occurring complex bursts of longer duration that may represent the lung inflation cycle. However, the latter pattern was not consistently present and therefore could not be studied in further detail. Interestingly, these complex bursts were never found under Cl^--free conditions suggesting that chloride ions may be necessary for their generation.

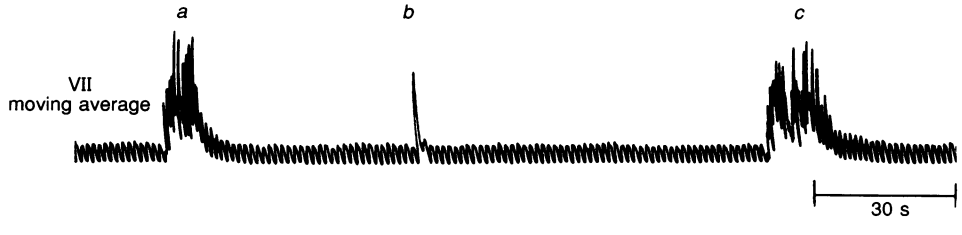

Figure 11. Examples of the presumed complex lung bursts (*a* and *c*) and a typical single lung burst (*b*) in a single animal

In contrast to a stable pattern of the typical short, sharp rising bursts for lung ventilation, the complex bursts were variable in form and duration. There was a clear oscillation of activity during complex bursts.

Different phases within both the lung and gill bursts can be identified (Sakakibara, 1984; Kogo & Remmers, 1994; Kogo *et al.* 1994; Liao *et al.* 1996). The details of the activity pattern within bursts are of interest when neural activity is related to the action of the complex mechanical apparatus that is involved in lung and gill ventilation. However, our study focused primarily on the effect of Cl^--mediated inhibition on fictive lung and gill rhythmicity. Therefore, we measured the frequency, amplitude and duration of the bursts as a whole rather than the changes in timing and amplitude of the various components of the bursts.

In the first series of experiments, we superfused the brainstem with the inhibitory neurotransmitters GABA and glycine. Both had profound depressant effects on lung and gill bursting rhythms and, at higher concentrations, both rhythms were abolished. Depressant effects of these agonists on respiratory motor output have been described previously, both for gill ventilation in lamprey (Rovainen, 1983) and pulmonary ventilation in mammals (Feldman & Smith, 1989; Hayashi & Lipski, 1992). We found that the neural output for gill bursts was abolished at lower concentrations of either agonist than was the neural output for lung bursts. Thus, the neural output for gill oscillations is more sensitive to inhibitory amino acids than that for lung bursts.

There may be multiple explanations for this finding. Respiratory motoneurons in amphibians presumably have membrane receptors for both GABA and glycine because they receive a variety of phasic, Cl^--mediated inhibitory inputs (Kogo & Remmers, 1994; Liao *et al.* 1996). A majority of VII motoneurons are modulated by both rhythms (Fig. 7 of Liao *et al.* 1996). Therefore, the simplest explanation is that gill bursting, which may be weaker than lung bursting activity, is more readily suppressed by inhibitory action exerted at the motoneuronal level where there is convergence of these different inputs (Liao *et al.* 1996). However, superfusion with agonists not only produced changes in burst amplitudes, but also in the frequencies of both lung and gill bursts; these responses were concentration dependent with gill bursting being more sensitive. Thus, despite the possibility of effects exerted at the motoneuronal level, the results suggest that the inhibitory amino acids used exert a differential effect on the generators of the two rhythms.

Our studies with the specific $GABA_A$ receptor antagonist bicuculline and the glycinergic antagonist strychnine also suggest differential effects of these two transmitter systems on gill and lung rhythmicities, although non-specific excitatory effects of these antagonists, when administered individually, limit interpretation of these studies. However, in the experiments involving combined superfusions with both antagonists at concentrations which caused each one individually to produce non-specific convulsive excitatory effects, lung rhythmicity was maintained, while gill rhythmicity was abolished. Although the reason for this somewhat paradoxical lack of non-specific excitation with combined superfusions is not clear, the observation suggests the presence of sites where the combined action of strychnine and bicuculline have an anticonvulsant effect. The advantage of this finding for the present study is that it allowed us to demonstrate that lung rhythmicity, in contrast to the gill rhythm, persists despite pharmacological blockade of fast synaptic inhibition.

Our observations with combined bicuculline and strychnine superfusion were extended by studies in Cl^--free medium. Superfusion with Cl^--free solution produced an initial tonic excitation, presumably due to reversing the normal gradient for chloride across the neuronal membrane. Following this transition period, the rhythmic gill oscillations were abolished while the rhythm for lung ventilation persisted. Between the lung bursts there was tonic activity, thus excluding the possibility that a gill-related input was present centrally, but subthreshold to cause motoneuronal firing. The lung bursts generated in Cl^--free medium occurred with slightly slower frequency than in control conditions and were larger in amplitude and longer in duration. Their occurrence was also more regular than in the control conditions. When Cl^- was returned to the superfusate, there was initially a cessation of all rhythmic activity, again presumably as a result of a transient enhancement of the Cl^- gradient across the cell membrane. Subsequently, in some, but not all, preparations both rhythms were restored. Thus, both a simultaneous pharmacological blockade of $GABA_A$ and glycine receptors and removal of Cl^- from the medium eliminate gill rhythm. This result strongly suggests that the gill rhythm is generated by an oscillator that relies on inhibitory interaction between groups of neurons. Alternatively, however, there could be pacemaker cells whose membrane potential, when disinhibited, moves to a region where the cell's pacemaker property cannot be expressed. In either case, the effects on the gill rhythm are in sharp contrast to those on lung rhythmicity. The latter rhythm persists in the absence of Cl^--mediated inhibition with enhanced regularity. Thus, it is not critically dependent on Cl^--mediated inhibition, although this does play a role in shaping the normal lung burst and determining its duration. Indeed, direct evidence for Cl^--mediated inhibition at the VII motoneuron level during the lung burst is shown in the companion manuscript (Liao *et al.* 1996).

Previous studies in lamprey have examined the role of fast synaptic inhibition in respiratory (gill) motor output (Thompson, 1985; Russell, 1986). However, the results of our study of inhibitory mechanisms involved in the rhythmogenesis of gill rhythmicity can be best compared with the study of Rovainen (1983) in adult lamprey. Compatible with our results, Rovainen reported that superfusion of an isolated lamprey brainstem with GABA

or glycine reduced the frequency and amplitude of gill ventilatory bursts at lower concentrations, while, at high concentrations, the rhythm was abolished. In contrast to our results following blockade of fast synaptic inhibition with picrotoxin, strychnine, bicuculline, alone or in combination, or by replacement of Cl^-, the gill rhythm persisted, albeit with increased burst duration. These results have led Rovainen to conclude that fast synaptic inhibition is not essential for rhythmogenesis in gill ventilation. His results, however, were complicated, as ours were in certain conditions, with non-specific effects inducing 'seizure-like' activity. Moreover, Rovainen (1983) reported that in Cl^--free conditions the gill rhythm ultimately ceases. Thus, it is unclear to what degree our results are different to those of Rovainen. Differences between Rovainen's results in lamprey and our data from amphibian larvae could be explained by differences in the degree of maturity of the animals studied (see, however, discussion below) or by a major species difference.

Our results pointing to the relative unimportance of fast synaptic inhibition for the generation of a basic lung rhythm in developing amphibia are compatible with recent data obtained in the *in vitro* brainstem–spinal cord preparations of the neonatal rat. Lung rhythm, as measured by phrenic and/or hypoglossal nerve outputs, persists in Cl^--free medium (Feldman & Smith, 1989). Based on these observations, these authors have proposed that cells with pacemaker properties generate pulmonary ventilation in the neonatal rat. Using microsectioning techniques, the site of these putative pacemaker cells has been localized to the pre-Bötzinger complex located in the rostral ventro-lateral medulla (Smith, Ellenberger, Ballanyi, Richter & Feldman, 1991). The presence of oscillatory behaviour in these cells is dependent on their membrane potential and thus they were termed conditional pacemakers. Likewise, Onimaru *et al.* (1989) have described that the majority of cells, that they call pre-I (pre-inspiratory), continue to exhibit rhythmic firing when synaptic transmission is blocked by incubation of the isolated brainstem in a solution with low calcium and high magnesium ion concentrations. Onimaru & Homma (1987) have argued that these cells, which fire both before and after the inspiratory burst, generate the basic respiratory rhythm. However, recent data in the neonatal rat have challenged this concept (Loddo, Völker, Bolis-Seidenschwanz, Ballanyi & Richter, 1994).

In contrast to the studies in neonatal rat brainstem, in an *in situ* artificially perfused brainstem preparation of adult rat, blockade of synaptic inhibition by perfusion of bicuculline or strychnine produced an increase in amplitude and frequency of inspiratory bursts recorded from the phrenic and hypoglossal nerves while Cl^--free solution abolished rhythmic bursting activity (Hayashi & Lipski, 1992). These different results may reflect differences in the preparations employed (for detailed discussion see Feldman & Smith, 1994). Another explanation, however, is that the putative pacemaker mechanism is only operative at an early stage of development. Consistent with this possibility are major changes with development in the role of glycine in the generation of respiratory rhythm in rodent brainstem (Paton, Ramirez & Richter, 1994) and major developmental changes in the respiratory motor output occurring both in amphibian larvae (Galante *et al.* 1992) and in mammals (Farber, 1988; Cooke & Berger, 1990). Thus, it is conceivable that the mechanism of respiratory rhythmogenesis changes as a function of development. In particular, our study shows that gill rhythmicity, which becomes functional earlier in development than lung rhythmicity, seems to be dependent on synaptic inhibition while lung rhythmicity is not. This observation suggests that there may be a general pattern of development in which rhythmic systems originate in cells with pacemaker properties and then gradually transform into network-based oscillators. Further studies are needed to address this postulate; amphibia may be an ideal model for such studies.

In conclusion, we have found that a lung ventilatory rhythm persists when Cl^--mediated inhibition is blocked while gill rhythm is abolished. This raises the possibility that, at least at this stage of development, there may be pacemaker cells for lung rhythm in amphibia.

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