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THE EFFECTS OF TONIC LUNG INFLATION ON VENTILATION IN THE AMERICAN BULLFROG *RANA CATESBEIANA* SHAW

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Summary

This study was designed to determine whether lung inflation stimulates or inhibits breathing in frogs by examining the effect of tonic lung inflation on the 'fictive' breathing pattern of decerebrate, unidirectionally ventilated bullfrogs. Neural discharge was monitored in the trigeminal nerve as an indication of the frequency and force of contraction of the buccal pump, and in the laryngeal branch of the vagus nerve as an indication of glottal opening, and hence fictive lung ventilation. Based on the temporal coordination of discharge in the trigeminal and vagus nerves during naturally occurring breaths it was possible to characterize the fictive breaths as inflation, deflation or balanced breaths. Increasing lung inflation increased absolute breathing frequency by reducing the duration of apnea between breaths and promoting a change in breathing pattern from no breathing to single breaths, breathing episodes and, finally, to continuous breathing. Associated with this was a decrease in the amplitude and area of the integrated trigeminal electroneurogram associated with the lung breaths, indicative of a reduction in the force of the buccal pump, and a shift in the timing of the trigeminal and vagal discharge, indicative of a shift from inflation to deflation breaths. Taken together the data suggest that lung deflation produces infrequent, large-amplitude inflation breaths or cycles, but that progressive lung inflation changes the breathing pattern to one of high-frequency attempts to deflate the lungs that are largely passive, and accompanied by contractions of the buccal pump that are no larger than those associated with normal buccal oscillations.

Key words: frog, *Rana catesbeiana*, ventilation, breathing pattern, lung inflation, pulmonary stretch receptor, Hering–Breuer reflex.

Introduction

There are four motor patterns that make up the anuran breathing repertoire. (1) Buccal oscillations, which ventilate the oropharynx alone. The nares remain open while the floor of the buccal cavity moves up and down continuously, without ever forcing air into the lungs. This behaviour has been speculated to serve an olfactory function (Foxon, 1964) or to keep the buccal cavity ventilated with fresh air, ensuring that subsequent lung ventilations will have ample oxygen content (De Jongh and Gans, 1969). (2) The 'typical' breath exhibited in resting anurans is thought to be a balanced lung ventilation in which pulmonary pressure and volume return to roughly similar levels at the end of each ventilation cycle (De Jongh and Gans, 1969; Vitalis and Shelton, 1990). (3) Under conditions of high respiratory drive, multiple breaths may occur in rapid succession without allowing time for lung emptying. Such lung inflation cycles are characterized by progressive increases in air pressure and volume in the lungs throughout the cycle (West and Jones, 1975; Macintyre and Toews, 1976; Vitalis and Shelton, 1990). (4) Lung deflation cycles have not been studied extensively (Vitalis and Shelton, 1990), but consist of a series of breaths in which air pressure and volume in the lungs are progressively reduced.

It is currently believed that, in mammals, phasic stimulation of slowly adapting pulmonary stretch receptors located throughout the tracheobronchial tree and lungs, as would occur with each breath, modulates the frequency of breathing by inhibiting further inhalation during the inspiratory phase or causing prolongation of exhalation during the expiratory phase (for reviews see Kubin and Davies, 1995; Tenney and Leiter, 1995). This is the Hering–Breuer reflex. Tonic stimulation of these same receptors, as would occur with changes in resting lung volume (or functional residual capacity), alters the timing of inspiration relative to expiration in a manner that acts to stabilize the resting lung volume and resist change (D'Angelo and Agostini, 1975; Muza and Frazier, 1983; Finkler and Iscoe, 1984; for review see Milsom, 1990). Vagotomy, the severing of the vagus nerve, eliminates these reflexes.

While it is generally believed that amphibians also possess a 'classic' Hering–Breuer reflex as seen in other vertebrates (for reviews see Tenney, 1979; Tenney and Leiter, 1995), the manner in which it is manifested must be significantly different. Since amphibians achieve pulmonary ventilation through a force–pump mechanism in which the lungs remain

inflated during the resting phase of the ventilation cycle, amphibians must not experience the same impulse for exhalation in response to phasic lung expansion as do mammals. The consequences of tonic lung inflation in amphibians, and particularly their reflexes, are also poorly understood and controversial.

Kogo et al. (Kogo et al., 1994) showed that in decerebrate, chemically paralyzed and unidirectionally ventilated (UDV) bullfrogs (*Rana catesbeiana*), breathing frequency (as determined by the frequency of bursts of motor activity in the trigeminal nerve; fictive breathing) was decreased when all proprioceptive feedback from the lungs was removed through bilateral vagotomy. Kinkead and Milsom (Kinkead and Milsom, 1997), using a similar *in situ* preparation, also found that breathing frequency was decreased, both when proprioceptive feedback was abolished through bilateral vagotomy, and when the lungs were deflated. They also demonstrated that breathing frequency was increased when the lungs were inflated, but that the amplitude of the trigeminal motor output associated with fictive breaths in this preparation was inversely proportional to pulmonary stretch receptor feedback. When the pulmonary branch of the vagus nerve was artificially stimulated in *in vitro* brainstem preparations (Kinkead et al., 1994) to simulate pulmonary stretch receptor feedback for each fictive breath, fictive breath frequency also increased dramatically, suggesting that lung inflation should stimulate breathing (Kinkead and Milsom, 1997). These results, suggesting that lung inflation stimulates breathing and that lung deflation inhibits breathing in reduced preparations, are supported by several other studies (McLean et al., 1995a; McLean et al., 1995b; Kinkead and Milsom, 1996; Reid and Milsom, 1998).

On the other hand Reid et al. (Reid et al., 2000), using *in situ* preparations of *Bufo marinus,* found that while lung deflation promoted episodic breathing, lung inflation promoted not only small, continuous lung ventilations at higher levels of respiratory drive, but also periods of apnea at low levels of respiratory drive. Then, in a recent study using the same preparation, reducing lung volume increased the frequency of trigeminal nerve discharge and lung inflation reduced fictive breathing frequency (Wang et al., 1999). With higher levels of tonic inflation, the amplitude of neural discharge associated with lung inflation recorded from the mandibular branch of the trigeminal nerve was reduced and lung ventilations appeared to be replaced with buccal oscillations (Wang et al., 1999). Kogo et al. also showed that decerebrate, chemically paralyzed and unidirectionally ventilated (UDV) bullfrogs (*Rana catesbeiana*) increased their breathing frequency when their lungs were deflated (Kogo et al., 1994). These results, suggesting that lung inflation inhibits breathing and that lung deflation stimulates breathing, support earlier findings in *Rana* sp. and *Xenopus laevis* (De Marneffe-Foulon, 1962; Shelton and Boutilier, 1982).

Our study was designed to address this controversy. Does lung inflation stimulate or inhibit breathing in frogs? Based on the evidence to date, we hypothesized that lung inflation should stimulate a transition to the production of deflation breaths involving reduced motor activity rather than the conversion of lung breaths to buccal oscillations. Using simultaneous recordings of electroneurograms (ENGs) from the mandibular branch of the trigeminal nerve (V_m, as an indication of the frequency and force of contraction of the buccal pump), and the laryngeal branch of the vagus nerve (X_l, as an indication of glottal opening, and hence fictive lung ventilation), in conjunction with changes in lung pressure, we monitored the responses to changes in tonic lung volume and pressure under conditions of high and low CO_2-related respiratory drive, to determine whether this was so.

Materials and methods

Housing

These experiments were conducted on six American bullfrogs, *Rana catesbeiana* (Shaw, 1802) of both sexes, weighing between 189 g and 467 g (mean mass=348 g), obtained from a commercial supplier (Charles D. Sulivan Company). The frogs were housed indoors at room temperature (21±1.5 °C) in fiberglass basins equipped with plastic platforms to hide beneath or bask upon. The basins were filled with dechlorinated water to a height of 8.5 cm, which was flushed daily. The frogs were fed live desert locusts (*Locusta migratoria*), crickets (*Acheta domestica*) or dew worms (*Lumbricus terrestris*) once per week, and were maintained with a daily photoperiod of 12 h:12 h L:D, with the light phase from 7:00 am to 7:00 pm.

Decerebration

A reduced frog preparation comparable to that developed by Kogo et al. (Kogo et al., 1994) was used in this study. Before surgery, the frogs were quarantined for at least 48 h following their last feeding to prevent complications. They were anaesthetized by submersion in a 1.5 g l^{-1} aqueous solution of MS-222 (3-aminobenzoic acid ethyl ester), pH 7.0 (balanced with sodium bicarbonate to neutrality), until there was no response to toe pinching and no eye-blink reflex (approx. 30 min). An incision was made along the sagittal plane on top of the head, between the eyes, towards the back of the skull. Using a Dremmel® moto-tool, a hole was then drilled into the skull, centered between the frontoparietal plates at a point slightly posterior to where they fuse rostrally to the exoccipital bones, to allow access to the brain. An incision was made transversely through the thalamus, between the cerebral hemispheres and the optic lobes, and the telencephalon was subsequently removed by suction, thereby eliminating cognitive function, pain perception and all higher cortical influences on breathing. The vacant cranium was then packed with cotton pellets and small (2–3 mm^3) pieces of Gelfoam® sterile sponge to promote clotting and maintain cranial pressure. The cranial opening was sealed with Vaseline, and a small piece of dental dam was affixed to the skull with Krazy Glue® to prevent water from entering the cranium. Following this, the skin was closed with sutures. The frogs were then

allowed to recover from the surgery for at least 48 h before further experimentation ensued.

Experimental set-up

On the day of an experiment, a small incision (1 cm) was made through the body wall on either side of the abdomen just anterior to the rear limbs. Through these openings, both lungs were cannulated with polyethylene tubing (PE 240) at their apical ends to allow unidirectional ventilation of the respiratory system (air flow into one lung and out of the other). Another incision was made through the body wall more anteriorly into one lung to accommodate a third catheter of polyethylene tubing (PE 80) to allow measurement of the internal lung pressure with a strain-gauge pressure transducer. Following insertion into the pulmonary lumen, the catheters were secured in place with 5-0 silk sutures. The skin and the hypaxial muscles of the body wall were then sutured closed separately around the catheters.

The frog was then placed on its back and an incision was made starting behind the margin of the mouth on one side and carried anteriorly along the mandible to the chin. The skin was retracted so that the mandibular branch of one trigeminal nerve (V_m) was located and isolated from the surrounding fascia. A piece of 5-0 silk was tied around the nerve before it was cut distally. Placing the frog on its abdomen, a dorsal approach was used to locate the vagus nerve on one side. The skin was cut transversely just anterior to the scapula and longitudinally along the spinal column. The fascia and muscles attached to the suprascapula were severed and the suprascapula retracted. The laryngeal branch of the vagus nerve (X_l) was located, isolated from surrounding fascia, and severed as far distally as possible.

To minimize movement during the experiment, the sciatic nerves innervating the rear limbs were severed in the region of the pelvic joint, and the brachial nerve on the same side as the exposed trigeminal nerve was also severed. This procedure was chosen over paralysis since our protocol (see below) required that animals be able to produce spontaneous inflations/deflations. The tympanic membranes were slit and the frog was then further immobilized by being placed in a stereotaxic head restraint.

Experimental procedures

Frogs were unidirectionally ventilated (UDV) with mixed gases administered from a Cameron GF-3/MP gas-mixing flowmeter at a rate of up to 500 ml min^{-1}. The gas was humidified by being bubbled through water in an Erlenmeyer flask before being conveyed to the inflow catheter of the unidirectional ventilation system. The degree of inflation of the lungs was regulated by controlling the resistance of the exhaust catheter by immersing its free end in a beaker of water. Unidirectional ventilation of the frogs commenced 1 h before the experiment began.

The isolated branches of the vagus and trigeminal nerves (X_l and V_m, respectively) were each placed on bipolar platinum hook electrodes and covered with a 1:1 mixture of Vaseline and mineral oil to protect against desiccation. Electrical nerve activity recorded from the bipolar electrodes was amplified and filtered, full-wave rectified and integrated (Gould) in 375 ms intervals. The ENGs were viewed on an oscilloscope and recorded on a polygraph and on computer (WinDaq™ version 1.32 data-acquisition system; DI-205, DATAQ Instruments) sampling at a frequency of 400 Hz per channel.

The pressure catheter was connected to a strain-gauge pressure transducer and the signal from this transducer was amplified and also recorded on the polygraph and computer data-acquisition system.

Experimental protocol

This experiment investigated the effects of changes in tonic lung inflation under varying degrees of CO_2-related respiratory drive. The frogs were placed on UDV, as described above, with gas mixtures containing one of six different levels of CO_2 in air (air, 1 %, 2 %, 3 %, 4 % and 5 % CO_2). The gas mixtures were administered in a random order. Once respiratory drive was established at each level of inspired gas, lung volume was set at different levels by immersing the UDV outflow catheter in a beaker of water to manipulate the outflow resistance of the UDV exhaust and thereby control lung pressure (P_L). Pulmonary pressure was initially set at 0 cmH_2O (1 cmH_2O= 98.1 Pa) and then subsequently increased in 1 cmH_2O increments at 5 min intervals to a maximum resistance of 5 cmH_2O.

To acquire data representative of deflation breaths, frogs were inflated to 5 cmH_2O with air and then the lung cannulae were occluded and UDV terminated to allow the frog to breathe as naturally as this preparation would permit. Similarly, for inflation breaths, UDV was stopped, the cannulae were opened to allow deflation of the lungs, and the cannulae subsequently closed to allow the frog to breathe more naturally. Balanced-breath data were acquired by terminating UDV, closing the cannulae, and allowing the frog to breathe on its own for 5 min in order to establish equilibrium.

Analytical procedures

Breathing frequency was quantified by analyzing the number of trigeminal (V_m) bursts that had corresponding vagal (X_l) bursts (a fictive breath) per unit time (termed absolute frequency; $f_{L,abs}$) as well as by measuring the period between each two successive, uninterrupted, integrated V_m bursts of discharge within any lung ventilation episode. The period was measured from the beginning of the onset of the first integrated V_m ENG discharge to the beginning of the subsequent integrated V_m ENG discharge. The inverse of this period was then multiplied by 60 to obtain the instantaneous frequency of breathing within an episode, $f_{L,inst}$.

The amplitude and area of V_m ENG discharge were recorded as a measure of respiratory effort and approximate tidal volume (Sakakibara, 1984b). Values for V_m amplitude were taken from the peak of the integrated ENG discharge (V s). The area of V_m discharge was the area under the trace of the integrated signal, measured from the onset of the fictive breath to its termination (V s^2).

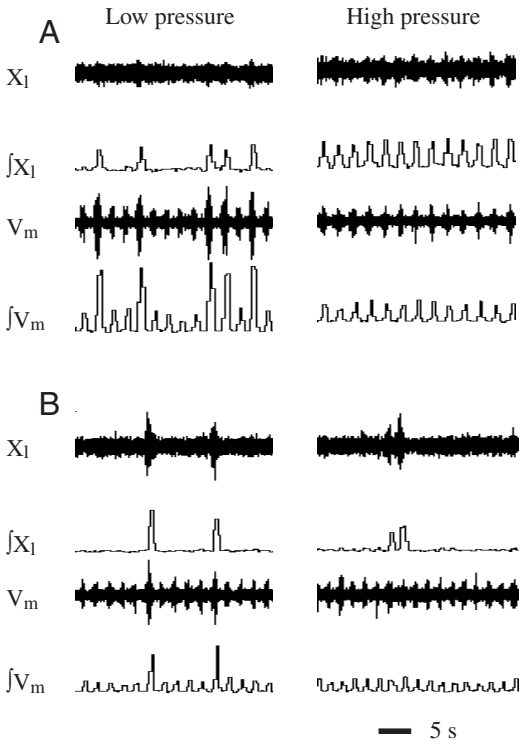

Fig. 1. Recordings of raw and integrated (∫) electroneurograms from the laryngeal branch of the vagus nerve (X_l) and the mandibular branch of the trigeminal nerve (V_m) in two animals with low (1 cmH_2O inflation pressure) and high [5 cm H_2O inflation pressure (A); 3 cmH_2O inflation pressure (B)] degrees of lung inflation while ventilated with 3 % CO_2.

Apnea was subjectively defined as a period between two fictive breaths, with a duration of at least two fictive buccal oscillations. Apnea duration was measured from the end of the last integrated V_m ENG discharge of an episode to the onset of the first integrated V_m ENG burst of the subsequent episode. Breath episodes longer than 1 min in duration with no apparent apneas were subjectively deemed to be continuous breathing.

For natural breaths (non-UDV), only the first fictive breath of an inflation or deflation breath series was analyzed since these were the breaths most strongly representative of an inflation or deflation pattern, respectively.

Data analysis

Values for fictive breathing variables were obtained by analyzing the events recorded during the last minute of exposure to a particular sequence (specific CO_2 content and outflow resistance) before the setting was changed. All data are presented as means ± S.E.M. The results were analyzed statistically using one-way repeated-measures analysis of variance (RM ANOVA; $P<0.05$) followed by a Student–Newman–Keuls test ($P<0.05$) to discern the statistical relevance of responses to changes in pressure at each gas setting, and two-way RM ANOVA ($P<0.05$) to determine the overall statistical relevance of changing each variable, taking into account the effects of the other variable.

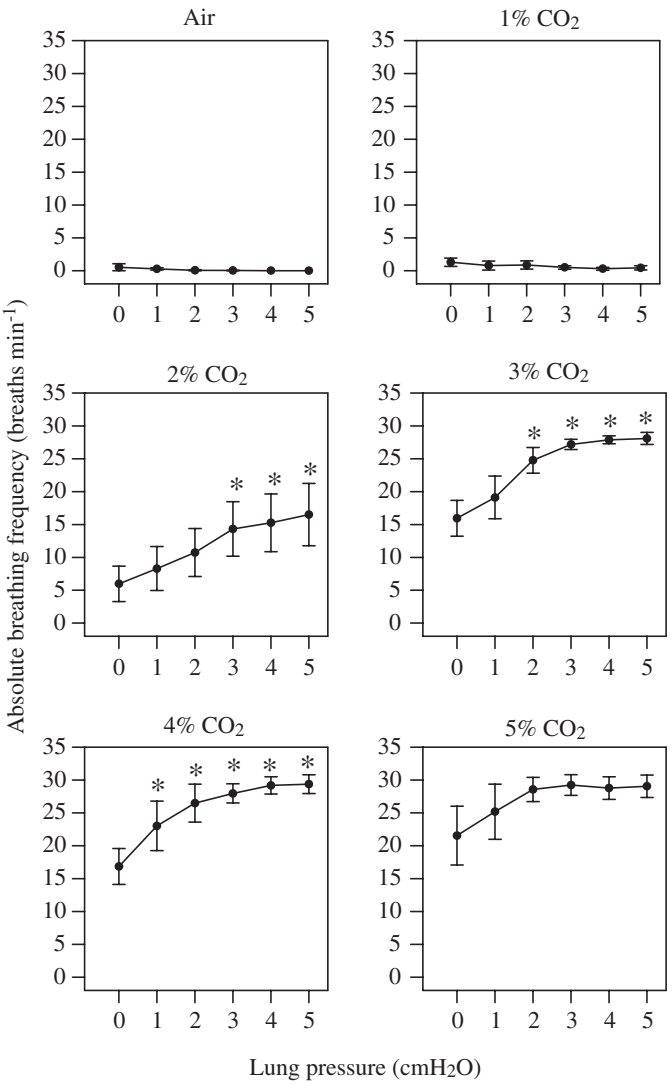

Fig. 2. The effects of tonic changes in lung pressure and inspired CO_2 content on absolute breath frequency ($f_{L,abs}$) of unidirectionally ventilated *Rana catesbeiana* (*$P<0.05$ relative to values at 0 cmH_2O). Values are means ± S.E.M. ($N=6$).

Results

Changes in the breathing pattern of bullfrogs in response to different levels of inflation and respiratory drive

Fig. 1 demonstrates the effects of tonic lung inflation upon the raw and integrated electroneurogram recordings from the trigeminal (V_m) and vagus (X_l) nerves. Under low CO_2-related respiratory drive and low lung pressure, breaths (indicated by simultaneous bursts of discharge in both V_m and X_l) occurred singly or in small episodes, with small-amplitude bursts of discharge (fictive buccal oscillations) occurring in V_m during the non-ventilatory period between breaths. Note that at increased levels of lung inflation, X_l amplitude was unchanged but that V_m burst amplitude was suppressed to the point that fictive lung ventilations in V_m became indistinguishable from fictive buccal oscillations. Fig. 1A shows an example where continuous breathing was evoked at higher degrees of lung

inflation with the magnitude of the motor output in V_m becoming comparable to that of the fictive buccal oscillations observed from V_m at lower levels of lung inflation. Fig. 1B demonstrates an example where although fictive lung ventilation frequency was not altered by lung inflation, the amplitude of the motor output in V_m became indistinguishable from that associated with fictive buccal oscillations. This figure serves to point out the importance of obtaining recordings from both V_m and X_I for distinguishing between lung breaths and buccal oscillations.

Absolute breathing frequency ($f_{L,abs}$) increased dramatically with increasing inspired CO_2 content (Fig. 2). Unidirectional ventilation at low levels of CO_2 frequently abolished breathing altogether, with higher levels of inspired CO_2 increasing fictive breath frequency (P<0.001). Increasing lung pressure also caused an increase in absolute frequency, with an apparent limit close to 30 breaths min^{-1} (P<0.001). There was a significant interaction between gas and pressure with respect to absolute breath frequency (P<0.001). Increased breathing frequency was associated with a decrease in the period of apnea between breaths or breathing episodes. Increasing levels of inspired CO_2 suppressed apnea duration (P<0.001) more than increasing lung inflation (P=0.003) (not illustrated). The instantaneous frequency of fictive breathing was more variable at lower levels of lung inflation than at higher levels of lung inflation, but a distinctive increase in instantaneous frequency ($f_{L,inst}$) was evident in response to increasing pulmonary pressure (P<0.001) (Fig. 3). However, increasing the level of inspired CO_2 had no significant effect upon instantaneous breathing frequency (P=0.09).

The effects of altering respiratory drive and lung inflation on the amplitude and area of the integrated trigeminal signal (used as indications of the volume of the fictive breath) were virtually identical and thus only the effects on amplitude are shown here (Fig. 4). Neither integrated V_m amplitude (P=0.96) nor area (P=0.95) was significantly affected by increased CO_2 levels (there were insufficient data points with air and 1 % CO_2 for inclusion of these treatments in the calculations), but integrated V_m amplitude (P=0.02) and area (P=0.006) were both significantly suppressed by increasing lung pressure. There was no statistically significant interaction between pulmonary pressure and inspired CO_2 content (P=1.000).

Increases in CO_2-related respiratory drive and lung pressure altered the coordination of trigeminal and vagal nerve discharges in the bullfrog. The timing of discharge in X_I in relation to V_m discharge was affected similarly by changes in lung pressure (P<0.001) and inspired CO_2 (P<0.001). Vagal nerve discharge began predominantly after trigeminal nerve discharge at lower levels of inspired CO_2 with concomitant low levels of lung inflation (Fig. 5). An increase in either of these stimuli (P_L or F_{ICO_2}) shortened the period between X_I and V_m discharge so that discharge in the two nerves occurred simultaneously, or nearly so. Further increase in P_L or F_{ICO_2} resulted in a switch in the timing so that V_m discharge began predominantly after X_I discharge at higher levels of lung inflation or inspired CO_2 (i.e. the delay in the onset of X_I discharge relative to the onset of V_m discharge was negative).

The breathing pattern displayed by the bullfrogs was also affected by afferent feedback (Fig. 6). When unidirectionally ventilated with air, breathing was abolished in most frogs, with only a few frogs exhibiting any breathing at lower pressures. As CO_2 content was increased, increasing proportions of frogs showed increasing amounts of respiration with the pattern progressing from no breathing, to single breaths, to episodic breathing, and finally to continuous breathing at higher respiratory drives. Increasing the degree of lung inflation had

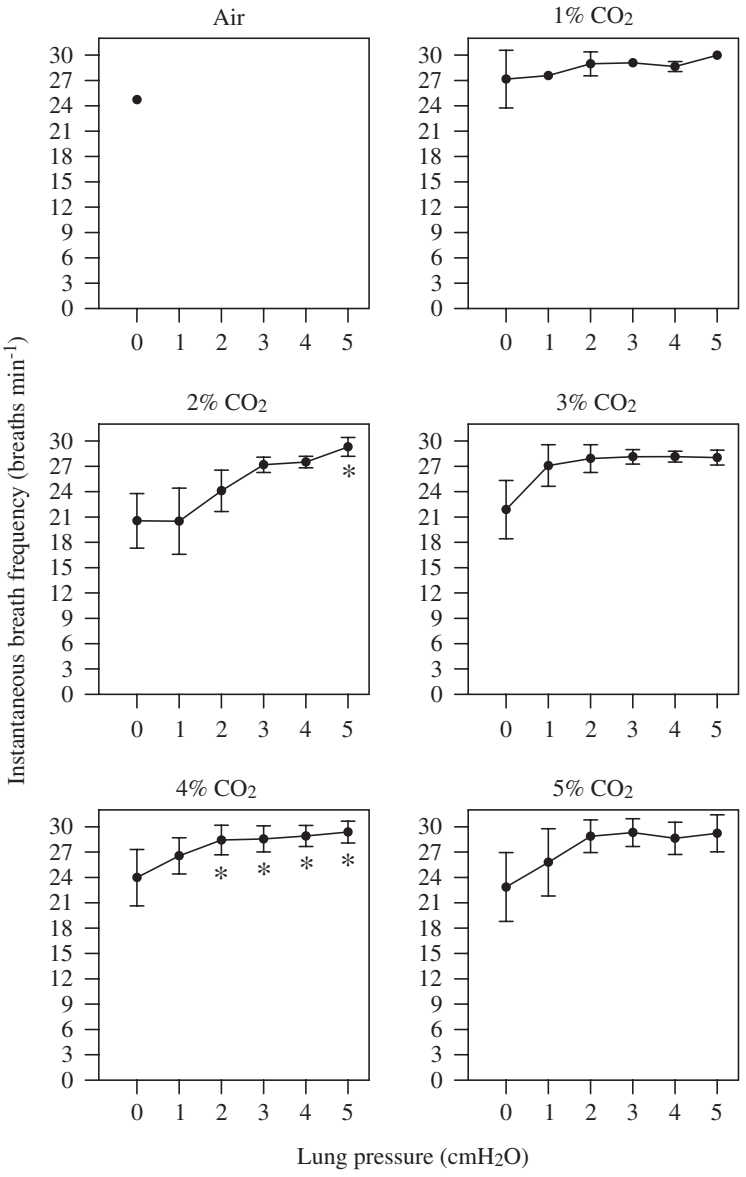

Fig. 3. The effects of tonic changes in lung pressure and inspired CO_2 content on instantaneous breathing frequency ($f_{L,inst}$) of unidirectionally ventilated *Rana catesbeiana* (*P<0.05 relative to values at 0 cmH_2O). Values are means ± S.E.M. (N=6).

a similar effect. Each animal showed a specific breathing pattern under a given set of conditions (pressure and level of CO_2) and Fig. 6 illustrates the predominant pattern for the group under each set of conditions.

Quantitative analysis of deflation, balanced and inflation breaths

The sequences of neural discharge in the trigeminal and vagus nerves were reversed for deflation cycles compared to inflation cycles (Fig. 7A). When efforts to inflate the lungs were stimulated by collapse of the lungs ($P_L \approx 0\,cmH_2O$), the onset of V_m discharge typically preceded the onset of X_l discharge by 0.33±0.18 s (Fig. 7). By contrast, when the lungs were inflated to P_L=5 cmH_2O with air, in the initial deflation cycle V_m discharge followed X_l discharge by 0.32±0.09 s (i.e. the delay in the onset of X_l discharge in relation to the onset of V_m discharge was negative; Fig. 7). During balanced breaths X_l and V_m discharge were more closely aligned, with the onset of V_m discharge typically 0.05±0.02 s after the onset of X_l discharge (Fig. 7B).

In this context, Fig. 8 demonstrates that increasing tonic lung pressure leads to both a decrease in the amplitude of the V_m burst discharge associated with each fictive breath and an increase in the delay between the onset of discharge in V_m compared to X_l. The mean values for the difference in onset of V_m and X_l discharge derived from the breathing sequences where lung volume and pressure were allowed to change (i.e. where lung inflation and deflation could be confirmed; Fig. 7), were then used to interpret the mean values recorded from frogs during the experiments where tonic lung pressure and volume were regulated. It can be seen in Fig. 9 that as F_{lCO_2} increases, frogs switch from not breathing or breathing with inflation cycles at low P_L, to balanced breaths and then to deflation breaths at elevated P_L.

Discussion

Pulmonary mechanics and fictive breathing

One of the great advantages of using 'reduced preparations' for the study of respiratory physiology is the ability to manipulate accurately the degree and pattern of lung filling independently of lung and arterial blood gases and *vice versa*. One drawback is the difficulty of interpreting respiratory behaviour from select recordings of neural motor output to the respiratory muscles. Ventilation in anuran amphibians is produced by a positive force pump, comprising the bucco–pharyngeal musculature (responsible for raising and lowering the floor of the mouth or buccal cavity) working in concert with glottal dilators, glottal constrictors and narial valves (De Jongh and Gans, 1969; Gans et al., 1969; Macintyre and Toews, 1976; Jones, 1982; Gans and Pyles, 1983). Each breath is characterized by four phases (Vitalis and Shelton, 1990). With the mouth and glottis closed and maxillar rims sealed, a typical lung ventilation begins with opening of the nares and lowering of the buccal cavity floor. This draws air through the nares and oral cavity into the buccal cavity (phase 1, buccal inspiration). The glottis, located in the fundus of the oral cavity, then opens through contraction of the laryngeal dilator muscles, permitting air from the lungs to pass through the buccal and oral cavities and out of the choanae (phase 2, lung emptying or 'expiration 1'). Lung inflation then occurs as the nares close and the buccal cavity floor is elevated, forcing the air in the distended buccal cavity through the glottis and into the lungs (phase 3, lung filling). As pressure in the buccal cavity begins to fall near the end of contraction of the oropharynx, the nares slowly open

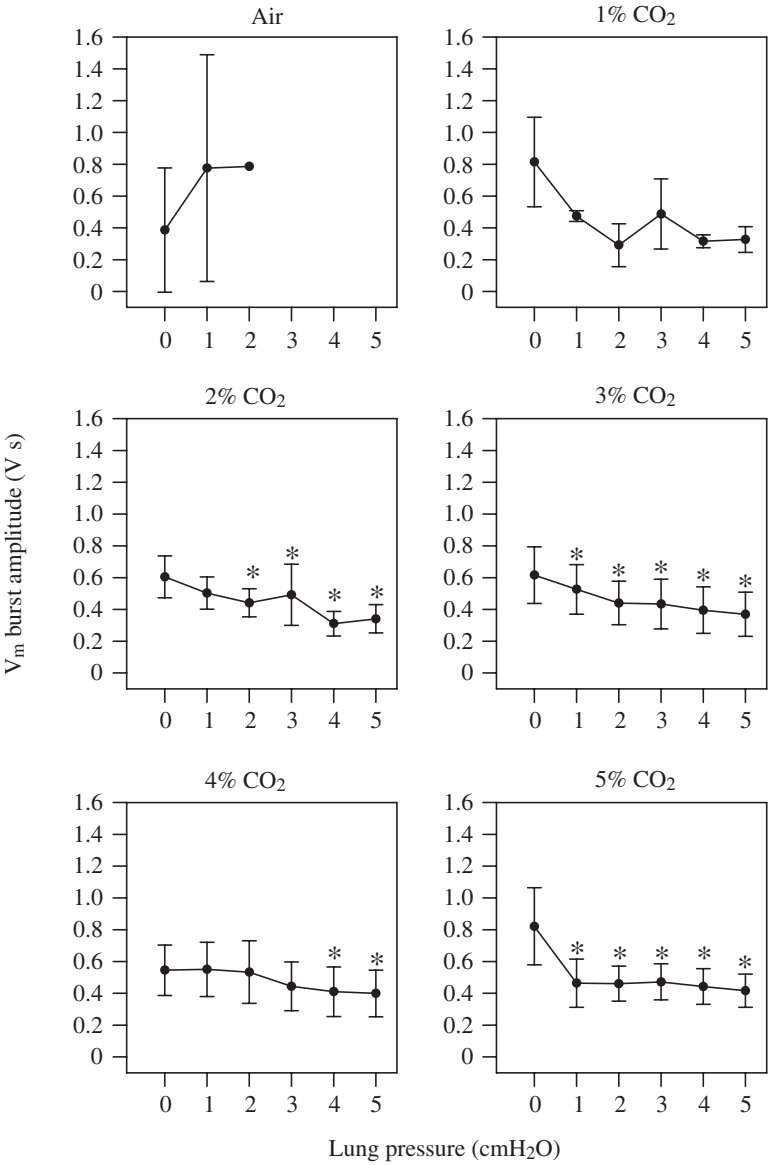

Fig. 4. The effects of tonic changes in lung pressure and inspired CO_2 content on the amplitude of integrated trigeminal nerve (V_m) discharge of unidirectionally ventilated *Rana catesbeiana* (*P<0.05 relative to values at 0 cmH_2O). Values are means ± S.E.M. (N=6).

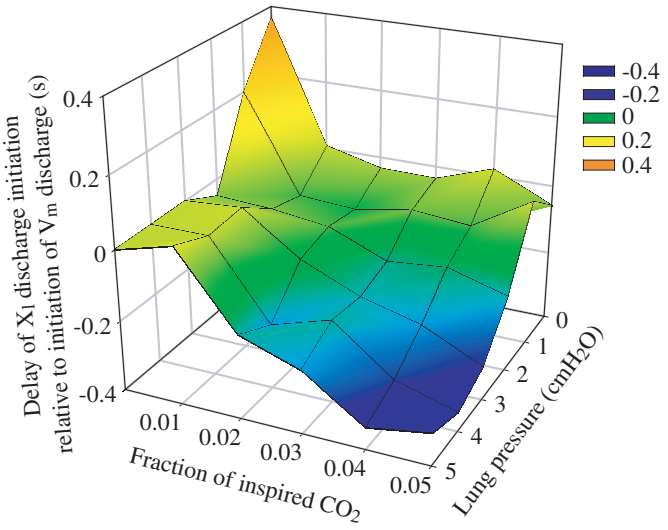

Fig. 5. The effects of tonic changes in lung pressure and inspired CO_2 content on the temporal coordination of trigeminal (V_m) and vagal nerve (X_I) discharge of unidirectionally ventilated *Rana catesbeiana*. Note that positive values for the delay indicate that the vagal discharge began after the trigeminal discharge, while negative values indicate that the vagal discharge was initiated first. Values are means ± S.E.M. (*N*=6).

and air in the lungs becomes trapped with the closure of the glottis. Residual air in the buccal cavity is pumped out through the now open nares (phase 4, buccal expiration or 'expiration 2') (West and Jones, 1975; Vitalis and Shelton, 1990). The lungs remain inflated in anurans during the inter-breath interval.

Whereas all breaths in mammals consist of a single, tidal cycle (inspiration–expiration–pause), there are four standard cycles that make up the anuran breathing repertoire, each having its own specific characteristics: buccal oscillations, balanced lung ventilations, lung inflation cycles, and lung deflation cycles. These are distinguished on the basis of mechanical events and the challenge has been to provide a basis for correlating fictive breathing with these behavioural events. Amalgamating data from the literature with the analysis of trigeminal (V_m) and laryngeal vagal (X_I) motor outputs obtained in the present study, however, the following picture emerges.

Buccal oscillations ventilate the oropharynx alone. The nares remain open while the floor of the buccal cavity moves up and down continuously, without ever forcing air into the lungs (De Jongh and Gans, 1969). With fictive breathing, such events can only be distinguished accurately from small lung ventilations in the presence of simultaneous evidence to indicate that the glottis remains closed (such as an absence of discharge in the laryngeal branch of the vagus nerve).

Balanced lung ventilations are thought to be the 'typical' breath exhibited in resting anurans (De Jongh and Gans, 1969; West and Jones, 1975; Sakakibara, 1984a; Sakakibara, 1984b; Shelton and Vitalis, 1990; Kogo et al., 1994; Kinkead et al., 1994; Kinkead and Milsom, 1996). In these breaths, the glottis opens following buccal expansion and lung deflation is immediately followed by inflation with an equal volume of air. Our data suggest that buccal compression commences soon after glottal opening (0.05±0.02 s) and thus there is little lag in the occurrence of motor output in V_m following that in X_I.

Either under conditions of high respiratory drive, or following lung deflation, multiple breaths may occur in rapid succession without allowing time for lung emptying. Such lung inflation cycles are characterized by an increase in the air pressure and volume in the lungs at the end of the cycle relative to that before the initiation of the cycle (West and Jones, 1975; Macintyre and Toews, 1976; Vitalis and Shelton, 1990; T. Baker and N. Smatresk, personal communication). Pulmonary pressure and lung volume generally increase in a 'ramp-like' manner with each breath of the cycle (West and Jones, 1975). There is a reduction in the lung-emptying phase of each breath and thus contraction of the bucco–pharyngeal musculature occurs earlier in the cycle, pushing air into the lungs before the air already in the lungs is able to leave (West and Jones, 1975; Vitalis and Shelton, 1990). West and Jones (West and Jones, 1975) report that occasionally the buccal floor is elevated before the glottis opens, indicating a shift in timing of the various motor outputs involved in ventilation, and our data confirm that trigeminal discharge (which would initiate contraction of the buccal musculature) generally occurs before

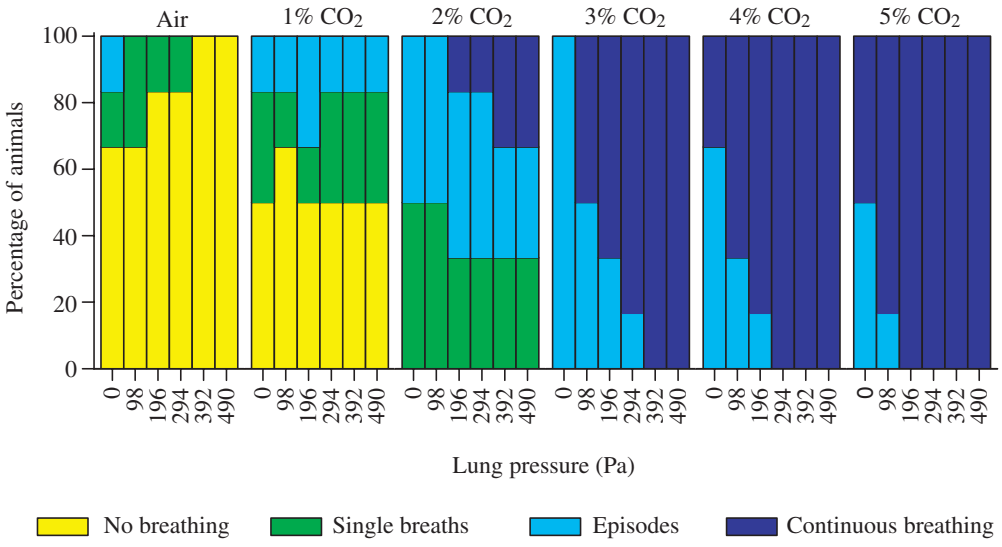

Fig. 6. The effects of tonic changes in lung pressure and inspired CO_2 content on the proportion of frogs exhibiting specific breathing patterns (*N*=6).

(0.33±0.18 s) the motor output in the laryngeal branch of the vagus nerve (which would initiate contraction of the glottal dilator muscles) with inflation cycles.

Lung deflation cycles have not been studied extensively. With the lung deflation cycle, air pressure and volume in the lungs at the end of the cycle is lower than it was at the onset of the cycle (Vitalis and Shelton, 1990; T. Baker and N. Smatresk, personal communication). Vitalis and Shelton report a prolongation of phases 1 and 2 (inspiration and lung emptying) before the onset of phase 3 (lung filling) in *Rana pipiens* during deflation breaths and our data confirm that under these circumstances, trigeminal discharge precedes the motor output in the laryngeal branch of the vagus nerve by 0.323±0.855 s.

From these data we can draw several conclusions. Fictive lung ventilations can be distinguished from fictive buccal oscillations by the presence of simultaneous discharge in the laryngeal branch of the vagus nerve (indicative of glottal opening) and the trigeminal nerve (indicative of buccal compression). By careful analysis of the timing of these two bursts of discharge relative to one another, these motor patterns can be further interpreted as events likely to produce deflation, balanced or inflation breaths. Given this, we can now more closely examine the effects of tonic lung inflation on fictive breathing patterns.

Changes in the fictive breathing pattern of bullfrogs in response to different levels of inflation and respiratory drive

Increasing lung inflation increased absolute breathing frequency by reducing the duration of apnea between breaths and promoting a change in breathing pattern, from no breathing to single breaths, breathing episodes, and finally to continuous breathing. Associated with this was a decrease in the amplitude and area of the integrated trigeminal electroneurogram associated with the lung breaths, indicative of a reduction in the force of the buccal pump. There was also a shift in the timing of the trigeminal and vagal discharge. Based on the temporal coordination of discharge in the trigeminal and vagus nerves during naturally occurring breaths as just described, we characterized fictive breaths as inflation, deflation or balanced breaths. Based on these interpretations, lung inflation also led to a shift from inflation to deflation

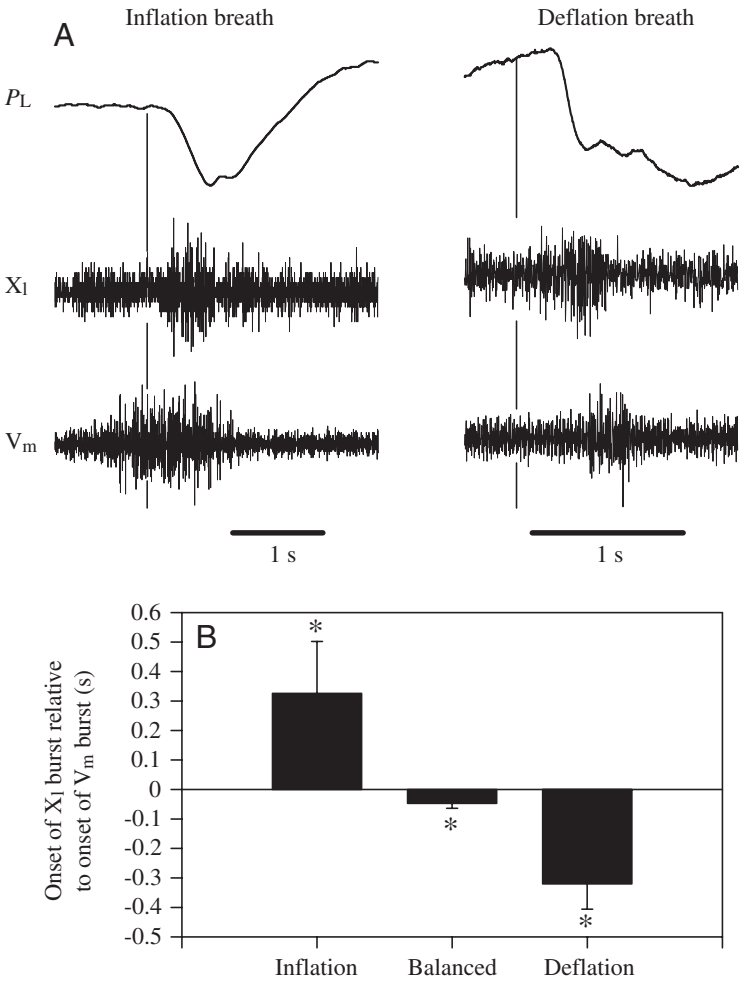

Fig. 7. (A) Recordings of lung pressure P_L and raw electroneurograms from the trigeminal (V_m) and vagus (X_l) nerves during an inflation breath (frog deflated to approximately 0 cmH_2O) and a deflation breath (frog inflated to 5 cmH_2O with air). Vertical bars indicate initiation of X_l discharge. (B) The onset of discharge (burst) activity in the vagus nerve relative to the onset of discharge (burst) activity in the trigeminal nerve during inflation, balanced and deflation breath cycles (*P<0.05 relative to 0). Values are means ± S.E.M. (N=6).

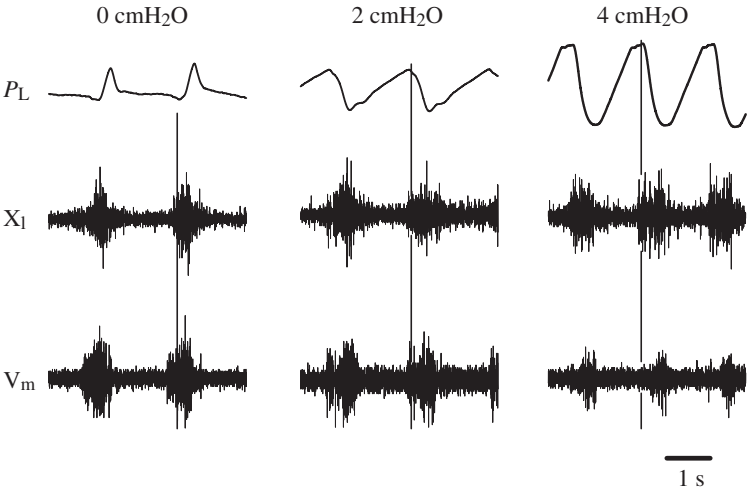

Fig. 8. Recordings of lung pressure (P_L) and raw electroneurograms from the trigeminal (V_m) and vagus (X_l) nerves during tonic inflation of the lungs to 0, 2 and 4 cmH_2O pressure (ventilated with 3% CO_2 in air). At 0 cmH_20, discharge in V_m commences (solid line) before discharge in X_l (inflation breath). At 2 cmH_2O, both nerves commence discharge at roughly the same time (solid line, balanced breath). At 4 cmH_2O, discharge in X_l commences (solid line) before discharge in V_m (deflation breath).

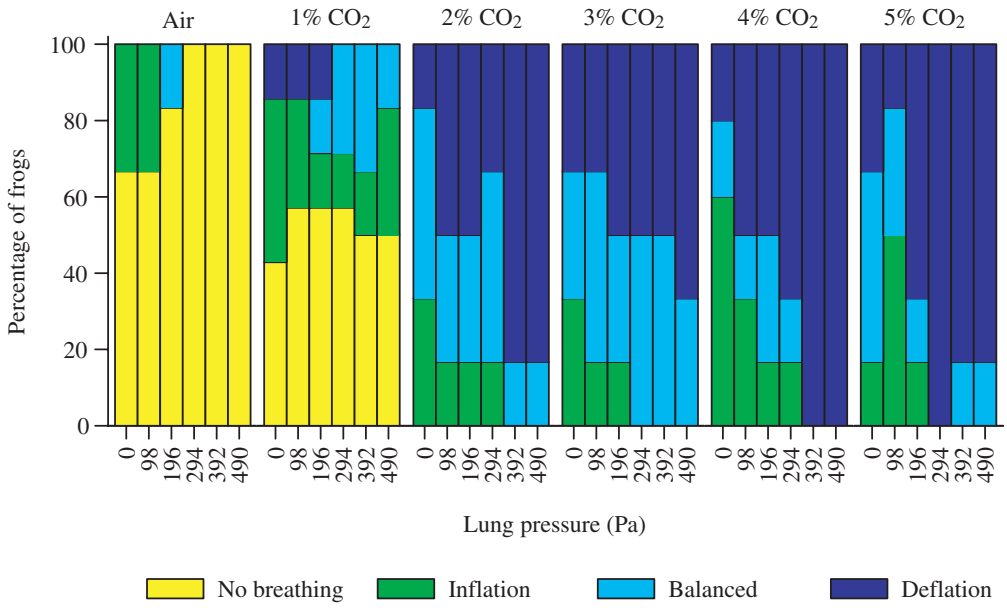

Fig. 9. Proportion of frogs (*N*=6) exhibiting inflation, balanced and deflation breaths under different degrees of lung inflation and respiratory drive. Based on information derived from Fig. 7.

breaths. Taken together the data suggest that lung deflation produces infrequent, large-amplitude inflation breaths or cycles. They also suggest that progressive lung inflation changes the breathing pattern to one of high-frequency attempts to deflate the lungs, which are largely passive and accompanied by contractions of the buccal pump that are not larger than those associated with normal buccal oscillations.

There has been some controversy surrounding the consequences of tonic lung inflation on breathing pattern in frogs and toads, which can possibly now be resolved in light of these findings. While one group of studies suggested that lung inflation stimulated breathing and that lung deflation inhibited breathing (Kinkead et al., 1994; Kinkead and Milsom, 1996; Kinkead and Milsom, 1997; Reid and Milsom, 1998), another group of studies suggested the opposite (De Marneffe-Foulon, 1962; Shelton and Boutilier, 1982; Wang et al., 1999) and a few studies obtained both results (Kogo et al., 1994; Reid et al., 2000). Invariably, in all studies, the amplitude of the trigeminal motor output associated with fictive breaths was inversely proportional to the volume of the lungs. Much of the controversy arises from interpretation of the high-frequency, low-amplitude bursts of motor output obtained from the trigeminal nerve with lung inflation. In some studies they were interpreted as small breaths, and hence as an increase in breathing frequency, while in other studies they were interpreted as buccal oscillations, and hence a reduction in breathing frequency. Based on the present study we conclude that the low-amplitude bursts of motor output seen in these previous studies were most likely a mix of small breaths and buccal oscillations and that the effect of the lung inflation was both inhibitory and excitatory; i.e. lung inflation inhibits large-amplitude inflation breaths and stimulates small-amplitude attempts to deflate the lungs. The remainder of the controversy may stem from differences in the level of respiratory drive present during lung deflation. While the data from the present study clearly show a transition from continuous breathing to breathing in episodes, single breaths and then apnea, as respiratory drive and lung volume are reduced, they also show that at low levels of respiratory drive (i.e. unidirectional ventilation with air), lung deflation leads to the reappearance of single breaths and, on rare occasions, some breathing episodes (Fig. 6). As a rule, these breaths were attempts to inflate the lungs (Fig. 9).

Biological significance

The emphasis of this study has been on the consequences of tonic changes in lung volume on breathing pattern. In mammals, where exhalation is relatively passive and the respiratory pause occurs at end-expiration, tonic lung inflation leads to a shortening of inspiration and a lengthening of expiration, which act to reduce lung volume. The net effect on respiratory frequency is generally a slowing or no change. In anuran amphibians, the respiratory pause occurs at end-inflation when the glottis closes, maintaining the lungs tonically in the inflated state. Our manipulations mimic this situation, and in this light the data suggest that increased lung volume leads to increased attempts to deflate the lungs. In both cases inspiratory efforts are reduced, expiration is promoted and the only real difference is the net effect on breathing frequency. This may be a necessary consequence of the differences in the respiratory pumps (aspiration *versus* force pump) and the position of the respiratory pause (end-expiratory *versus* end-inspiratory) in the ventilation cycle in the two groups of animals. In mammals, the pause occurs at end-expiration with the glottis open, allowing extended time for lung deflation to occur. In the frog, the pause occurs at end-inflation with the glottis closed, and extending the time available for deflation can only occur by increasing breathing frequency. It is interesting to note that increasing levels of inspired CO_2 tended to produce similar effects to lung inflation.

This may indicate that high levels of pulmonary CO_2, regardless of the degree of lung inflation, promote increased attempts to deflate the lungs, perhaps in an attempt to turn over and replenish lung contents.

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References

D'Angelo, E. and Agostini, E. (1975). Tonic vagal influences on inspiratory duration. *Respir. Physiol*. **24**, 287–302.

De Jongh, H. J. and Gans, C. (1969). On the mechanism of respiration in the bullfrog, *Rana catesbeiana*: a reassessment. *J. Morphol*. **127**, 259–290.

De Marneffe-Foulon, C. (1962). Contribution à l'etude du mechanisme et du controle des mouvements respiratoires chez *Rana*. *Annal. Soc. Roy. Zool. Belgique* **92**, 81–132.

Finkler, J. and Iscoe, S. (1984). Control of breathing at elevated lung volumes in anesthetized cats. *J. Appl. Physiol*. **56**, 839–844.

Foxon, G. E. H. (1964). Blood and respiration. In *Physiology of the Amphibia* (ed. J. A. Moore), pp. 151–209. New York, Academic Press.

Gans, C., De Jongh, H. J. and Farber, J. (1969). Bullfrog (*Rana catesbeiana*) ventilation: how does the frog breathe? *Science* **163**, 1223–1225.

Gans, C. and Pyles, R. (1983). Naral closure in toads: which muscles? *Respir. Physiol.* **53**, 215–223.

Jones, R. M. (1982). How toads breathe: control of air flow to and from the lungs by the nares in *Bufo marinus. Respir. Physiol*. **49**, 251–265.

Kinkead, R., Filmyer, W. G., Mitchell, G. S. and Milsom, W. K. (1994). Vagal input enhances responsiveness of respiratory discharge to central changes in pH/pCO_2 in bullfrogs. *J. Appl. Physiol*. **77**, 2048–2051.

Kinkead, R. and Milsom, W. K. (1996). CO_2-sensitive olfactory and pulmonary receptor modulation of episodic breathing in bullfrogs. *Am. J. Physiol.* **270**, R134–R144.

Kinkead, R. and Milsom, W. K. (1997). Role of pulmonary stretch receptor feedback in control of episodic breathing in the bullfrog. *Am. J. Physiol*. **272**, R497–R508.

Kogo, N., Perry, S. F. and Remmers, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. I. *J. Neurobiol*. **25**, 1067–1079.

Kubin, L. and Davies, R. O. (1995). Central pathways of pulmonary and airway vagal afferents. In *Lung Biology in Health and Disease,* Vol. 79*, Regulation of Breathing* (ed. J. A. Dempsey and A. I. Pack), pp. 219–284. New York, Marcel Dekker.

Macintyre, D. H. and Toews, D. P. (1976). The mechanics of lung ventilation and the effects of hypercapnia on respiration in *Bufo marinus*. *Can. J. Zool*. **54**, 1364–1374.

McLean, H. A., Kimura, N., Kogo, N., Perry, S. F. and Remmers, J. E. (1995a). Fictive respiratory rhythm in the isolated brainstem of bullfrogs. *J. Comp. Physiol.* **176**A, 703–713.

McLean, H. A., Perry, S. F. and Remmers, J. E. (1995b). Two regions in the isolated brainstem of the frog that modulate respiratory-related activity. *J. Comp. Physiol.* **177**A, 135–144.

Milsom, W. K. (1990). Control and co-ordination of gas exchange in air breathers. In *Advances in Comparative and Environmental Physiology,* Vol. 6. (ed. R. G. Boutilier), pp. 347–400, Berlin, Springer-Verlag.

Muza, S. R. and Frazier, D. T. (1983). Response of pulmonary stretch receptors to shifts of functional residual capacity. *Respir. Physiol*. **52**, 371–386.

Reid, S. G. and Milsom, W. K. (1998). Respiratory pattern formation in the isolated bullfrog (*Rana catesbeiana*) brainstem-spinal cord. *Respir. Physiol*. **114**, 239–255.

Reid, S. G., Milsom, W. K., Meier, J. T., Munns, S. and West, N. H. (2000). Pulmonary vagal modulation of ventilation in toads (*Bufo marinus*). *Respir. Physiol*. **120**, 213–230.

Sakakibara, Y. (1984a). The pattern of respiratory nerve activity in the bullfrog. *Japan. J. Physiol*. **34**, 269–282.

Sakakibara, Y. (1984b). Trigeminal nerve activity and buccal pressure as an index of total inspiratory activity in the bullfrog. *Japan. J. Physiol*. **34**, 827–838.

Shelton, G. and Boutilier, R. G. (1982). Apnoea in amphibians and reptiles. *J. Exp. Biol*. **100**, 245–273.

Tenney, S. M. (1979). A synopsis of breathing mechanisms. In *Lung Biology in Health and Disease,* Vol. 13. *Evolution of Respiratory Processes: A Comparative Approach* (ed. S. C. Wood and C. Lenfant), pp. 51–106. New York, Marcel Dekker Inc.

Tenney, S. M. and Leiter, J. C. (1995). The control of breathing, an unihibited survey from the perspective of comparative physiology. In *Lung Biology in Health and Disease,* Vol. 79, *Regulation of Breathing* (ed. J. A. Dempsey and A. I. Pack), pp. 3–36. New York, Marcel Dekker Inc.

Vitalis, T. Z. and Shelton, G. (1990). Breathing in *Rana pipiens*: the mechanism of ventilation. *J. Exp. Biol*. **154**, 537–556.

Wang, T., Taylor, E. W., Reid, S. G. and Milsom, W. K. (1999). Lung deflation stimulates fictive ventilation in decerebrated and unidirectionally ventilated toads (*Bufo marinus*). *Respir. Physiol*. **118**, 181–191.

West, N. H. and Jones, D. R. (1975). Breathing movements in the frog *Rana pipiens.* I. The mechanical events associated with lung and buccal ventilation. *Can. J. Zool*. **53**, 322–344.