The Journal of Experimental Biology 209, 1135-1146
Published by The Company of Biologists 2006
doi:10.1242/jeb.02106

Chronic hypercapnia modulates respiratory-related central pH/CO_2 chemoreception in an amphibian, *Bufo marinus*

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Accepted 24January 2006

Summary

Anuran amphibians have multiple populations of pH/CO_2-sensitive respiratory-related chemoreceptors. This study examined in cane toads (*Bufo marinus*) whether chronic hypercapnia (CHC) altered the pH/CO_2 sensitivity of central respiratory-related chemoreceptors *in vitro* and whether CHC altered the acute hypercapnic ventilatory response (HCVR; 5% CO_2) *in vivo*. Toads were exposed to CHC (3.5% CO_2) for 9 days. *In vitro* brainstem–spinal cord preparations were used to examine central respiratory-related pH/CO_2 chemosensitivity. CHC augmented *in vitro* fictive breathing as the pH of the superfusate was lowered from 8.2 to 7.4. Midbrain transection *in vitro* (at a level known to reduce the clustering of breaths) did not alter this augmentation. *In vivo*, CHC did not alter the acute HCVR but midbrain transection changed the breathing pattern and increased the overall level of ventilation. CHC did not alter the effect of olfactory CO_2 chemoreceptor denervation on the acute HCVR *in vivo* but did alter the response when returned to normal air. The results indicate that CHC increases the response of central pH/CO_2 chemoreceptors to changes in cerebrospinal fluid pH *in vitro* yet this increase is not manifest as an increase in the HCVR *in vivo*.

Key words: brainstem–spinal cord, central pH/CO_2 chemoreceptors, chronic hypercapnia, hypercapnic ventilatory response, olfactory CO_2 chemoreceptor, cane toad, *Bufo marinus*.

Introduction

Respiratory-related CO_2 control systems in amphibians are complex. As in other air-breathing vertebrates, anuran amphibians possess central pH/CO_2 chemoreceptors located on the ventrolateral surface of the medulla that increase breathing when stimulated by a decrease in cerebral spinal fluid pH (Smatresk and Smits, 1991; Taylor et al., 2003b). The peripheral chemoreceptors in the aortic body and carotid labyrinth (Macintyre and Toews, 1976; West et al., 1987) are stimulated by changes in arterial pH/CO_2, contributing approximately 25% of the increase in respiratory drive during acute hypercapnia (Smatresk and Smits, 1991; Van Vliet and West, 1992). Olfactory CO_2 chemoreceptors, located in the nasal epithelium, reduce breathing frequency when stimulated by increased levels of inspired CO_2 (Kinkead and Milsom, 1996; Coates, 2001; Milsom, 2002). Furthermore, pulmonary stretch receptor (PSR) discharge in anurans is CO_2 sensitive with increasing levels of CO_2 inhibiting PSR activity (Milsom and Jones, 1977; Kuhlman and Fedde, 1979). The effect of PSR feedback on breathing in anurans is complex in itself and depends upon whether or not the pattern of feedback is phasic or tonic and also on the state of lung inflation at the time of PSR feedback (Kinkead et al., 1994; Kinkead and Milsom, 1996; Wang et al., 1999; Wang et al., 2004; Reid et al., 2000b; Saunders and Milsom, 2001; Reid and West, 2004). Pulmonary vagotomy, which eliminates PSR feedback to the brain, abolishes the acute hypercapnic ventilatory response (Reid et al., 2000b).

Air-breathing animals are rarely faced with exposure to acute or chronic hypercapnia (CHC). Exceptions to this generalisation include those animals that live in underground burrows (Boggs et al., 1984; Kuhnen, 1986). Burrowing animals that live underground and breathe relatively high levels of CO_2 have, for the most part, a reduced ventilatory response to acute hypercapnia compared to non-burrowing species (Boggs et al., 1984). In temperate climates during the winter months, many terrestrial amphibians burrow into the soil (Pinder et al., 1992) or occupy burrows made by mammals. For example, the adult plains spadefoot toad (*Scaphiopus bombifrons*) (Russell and Bauer, 1993), the Manitoba toad (*Bufo hemiophrys*) (Breckenridge and Tester, 1961) and the Canadian toad (*Bufo hemiophrys*) will bury between 0.5 and 1.3 m during the winter while the Boreal toad (*Bufo boreas*) and the Great Plains toad (*Bufo cognatus*) may also over-winter in rodent burrows. Although measurements of O_2 and CO_2 levels in these temperate microhabitats have not been reported,

it is possible that these burrows also become hypoxic and hypercapnic.

Boutilier et al. (Boutilier et al., 1979b) reported elevated arterial P_{CO_2} levels (approximately 15 mmHg) in burrowing cane toads whose nares were open to the air while the skin was surrounded by sand at 25°C for 6 days. Boutilier et al. (Boutilier et al., 1979a) state that it is not uncommon for cane toads to encounter hypercapnic conditions in their environment, including hypercapnic waters in the tropics (Toews and Macintyre, 1978). Whereas the cardiorespiratory responses to acute hypercapnia in amphibians have been well studied, the effects of prolonged exposure to hypercapnia have not been addressed. A recent study, on cane toads, from this laboratory (McAneney et al., in press) demonstrated that exposure to chronic hypoxia blunts the acute hypoxic ventilatory response but does not alter resting levels of ventilation.

The goal of this study was to address the hypothesis that CHC would alter respiratory-related CO_2 chemoreceptor function and reduce the overall hypercapnic ventilatory response to a subsequent bout of acute hypercapnia. Cane toads (*Bufo marinus*; a species in which respiratory physiology has been well studied) were exposed to CHC for 9 days, following which central pH/CO_2 chemosensitivity was assessed using *in vitro* brainstem–spinal cord preparations and whole animal hypercapnic ventilatory responses were measured *in vivo*. A midbrain transection slightly caudal to the optic chiasma was previously demonstrated to reduce the clustering of breaths into episodes and cause an increase in fictive breathing frequency *in vitro* (Reid et al., 2000a). We hypothesised that a change in descending inputs, from the level of the optic chiasma, would alter CO_2 chemosensitivity following CHC. Furthermore, given that CHC would continually stimulate olfactory CO_2 chemoreceptors, we hypothesised that an increase in central pH/CO_2 chemosensitivity would occur to counter an increase in inhibitory input from olfactory chemoreceptors during CHC. Midbrain transection (*in vivo* and *in vitro*) and olfactory denervation (*in vivo*) experiments addressed these two hypotheses.

Materials and methods

Experimental animals

Cane toads (*Bufo marinus* L.) (N=91; 200–300 g) were obtained from a commercial supplier (Boreal Scientific, St Catharine's, Ontario, Canada) and maintained in a fibreglass tank at room temperature (20–22°C). Toads were supplied with both terrestrial and aquatic habitats consisting of moist peat moss and trays of dechlorinated water, respectively. The photoperiod was maintained at 12 h:12 h light:dark. Animals were fed live crickets once per week. Holding conditions and experimental protocols were approved by the University of Toronto Animal Care Committee and conform to the guidelines established by the Canadian Council for Animal Care.

Exposure to chronic hypercapnia

Toads were placed, for a 9 day period, into a Plexiglas chamber (35 cm×25 cm) within which the inspired CO_2 level was maintained at 3.5% using a Pro-CO_2 control unit (Biospherix, NY, USA). A CO_2 electrode, within the chamber, monitored the level of CO_2. When the CO_2 concentration fell below 3.5%, the Pro-CO_2 delivered a small amount of CO_2 to raise the level back to 3.5%. In this manner, the amount of CO_2 was maintained at a constant level within the chamber at all times. A level of 3.5% CO_2 was selected as it approximates the average CO_2 concentration found in mammalian burrows based on a study of 15 different species (Kuhnen, 1986). Routine measurements of O_2 (S-3A O_2 analyser, AEI, Pittsburgh, PA, USA) confirmed that inspired O_2 levels remained at approximately 20.2%. The chamber was maintained at room temperature and exposed to a 12 h:12 h light:dark cycle.

In vitro experiments

The in vitro brainstem-spinal cord preparation

Toads were anaesthetised by emersion in a solution of 3-aminobenzoic acid ethyl ester (MS222, 1.0 g l^{-1}; Sigma) neutralised with sodium bicarbonate. Animals were kept in the anaesthetic until the eye-blink and toe-pinch reflexes were eliminated. Using a Dremmel Tool, a longitudinal incision was made in the skull rostral to the optic lobes and the cranial case was removed with Rongeurs and bone shears, and placed onto a Sylgard-coated dissecting dish. The brain was exposed and superfusion with ice-cold oxygenated artificial cerebrospinal fluid (aCSF) was initiated; the rostral forebrain was then removed. The remaining brain tissue was continually superfused with the aCSF (in mmol l^{-1}; NaCl, 103; KCl, 4.05; $MgCl_2$, 1.38; glucose, 10; $NaHCO_3$, 29.2; $CaCl_2$, 2.45; pH 7.8) (Taylor et al., 2003a; Taylor et al., 2003b).

Cranial nerves were cut close to their exit to the skull and the spinal cord was severed at the level of the third spinal nerve. The preparation was transferred from the brain case and immobilised with insect pins in a Sylgard-coated dissecting dish continually superfused with oxygenated aCSF. The meninges surrounding the brain were removed in order to free the cranial nerve roots, and the nerve tips were cut to provide a clean surface for recording. The preparation was then pinned, ventral side up, onto a fine stainless steel mesh within a superfused recording chamber. The mesh divided the chamber into upper and lower compartments, which ensured simultaneous superfusion of both surfaces of the preparation (McLean et al., 1995). The preparation was continuously superfused with oxygenated aCSF, at a rate of 10 ml min^{-1}, using peristaltic pumps that delivered and removed the aCSF from the chamber. The aCSF was recycled. The preparations were maintained at pH 7.8 and room temperature for 60 min before commencing the experiment.

Suction electrodes of various diameters were made from thin-walled capillary glass (1 mm diameter) pulled to a fine tip using a vertical pipette puller (Kopf model 720, Tujunga, CA, USA). The tips were polished using a grinding stone and flame

to provide a smooth surface. Using a micro-manipulator, an appropriately sized suction electrode was positioned near the end of the vagus nerve root and the nerve was carefully aspired into the electrode such that a tight seal was obtained between the nerve and the electrode. In all preparations, recordings were taken of whole nerve discharge from the vagus nerve. In the intact animal, a branch of the vagus nerve innervates the glottis, which opens and closes with each breath. Since these preparations are devoid of any afferent input and breathing is an inherently rhythmic process generated in the brainstem, all rhythmic activity (*in vitro* motor output) recorded from the vagus nerve was assumed to represent motor output to the respiratory muscles (glottis) and is therefore an index of breathing termed fictive breathing (Sakakibara, 1984a; Sakakibara, 1984b; Kinkead et al., 1994; McLean et al., 1995; Reid and Milsom, 1998; Reid et al., 2000a; Reid et al., 2000b; Morales and Hedrick, 2002; Taylor et al., 2003a; Taylor et al., 2003b).

Nerve activity from the suction electrode was amplified (10×) and filtered (30 Hz, high pass; 3 kHz, low pass) using a DAM50 AC amplifier [World Precision Instruments (WPI), Sarasota, FL, USA]. The output from the DAM50 was sent to a second AC amplifier (ISO8A, WPI) and amplified a further 100×. The amplified nerve signal from the ISO8A was sent to a moving averager (CWE MA821/RSP) for integration (time constant=200 ms) and to an audio monitor (AM Systems Model 3300, Carlsborg, WA, USA). The amplified/filtered nerve signal and integrated trace were monitored and stored using a data acquisition system (Biopac Systems, MP150, Goleta, CA, USA). The sampling rate of the analogue to digital conversion was 2000 Hz.

Gassing the aCSF with varying levels of CO_2 (0–5%; balance O_2) altered the aCSF pH. The levels of CO_2 and O_2 gassing the aCSF were set using digital mass flow controllers (Smart-Trak 100, Sierra Instruments, Monterey, CA, USA). The pH level of the aCSF was monitored using a pH electrode placed within the aCSF reservoir.

Series 1: effects of CHC on pH-sensitive fictive breathing

Following the 1 h stabilisation period and the observation of stable levels of neural discharge (fictive breathing), the pH of the aCSF was lowered from 7.8 to 7.4. The pH was then raised by 0.2 units every 30 min until a pH of 8.2 was achieved. This pH range approximates that used in previous studies on amphibian brainstem–spinal cord preparations (e.g. McLean et al., 1995; Reid and Milsom, 1998). All experiments were performed at room temperature (approximately 22°C). This is within the temperature range (15–25°C) reported (Morales and Hedrick, 2002) in which fictive breathing is consistently active from *in vitro* adult bullfrog preparations. This protocol was performed on brains taken from control (N=6) and chronically hypercapnic (N=6) toads.

Series 2: effects of CHC and midbrain transection on pH-sensitive fictive breathing

This series used separate groups of toads from those used in series 1. The preparation was prepared as described above. Following the 1 h stabilisation period, the pH of the aCSF was increased to 8.0 and then lowered to 7.8 and 7.5 in 30 min intervals. Following this, the brain was transected slightly caudal to the optic chiasma at a level previously demonstrated to significantly attenuate (and almost abolish) episodic (clustered) fictive breathing (Reid et al., 2000a) as well as alter the pH sensitivity of fictive breathing in brains taken from chronically hypoxic toads (J. McAneney and S. Reid, unpublished data). Following a second 1 h stabilisation period, the pH changes (8.0, 7.8 and 7.5) were repeated. This protocol was performed on brains taken from control (N=8) and chronically hypercapnic (N=9) toads.

In vivo experiments

Series 3: breathing during the 9 days of CHC

In these experiments, breathing was measured by impedance as the per breath movement of the body wall. Toads were anaesthetised with MS222 as described above. Impedance leads, fabricated from thin insulated copper wire, were sutured to the flanks of the animal at the site of maximal displacement during lung inflation and deflation. We have previously validated impedance measurements as an appropriate measure of breathing in this species (McAneney et al., in press). Following a 48 h recovery period, toads were placed into the chronically hypercapnic chamber (described above) for 9 days. On each day of exposure to 3.5% CO_2, *in vivo*, breathing was recorded for 1 h and 15 min while the animals remained in the CHC chamber. To measure breathing, the impedance leads were connected to extensions which, in turn, input into an impedance converter (UFI, Morro Bay, CA, USA; model 2991). The signal from the impedance converter was recorded using a digital data acquisition system (DI 194; DataQ Systems, Akron, OH, USA) at a sampling rate of 120 Hz.

Series 4: effects of CHC and midbrain transection on the hypercapnic ventilatory response

Four groups of animals were studied in this series: (1) control, midbrain intact (N=9); (2) control, midbrain transected (N=8); (3) CHC, midbrain intact (N=8) and (4) CHC, midbrain transected (N=7).

Toads were anaesthetised and fitted with impedance leads as described above. In the midbrain-transected toads, following attachment of the impedance leads, a narrow opening was drilled in the skull slightly caudal to the optic chiasma. The brain was then transected using a sharp blade. The small hole was packed with cotton and covered with dental dam held in place by superglue. Following a 48 h recovery period the animals were placed into control or chronically hypercapnic conditions for 9 days. The surgical procedure used to open the skull in order to perform the *in vivo* brain transection is relatively minor and was performed at the same time as the impedance leads were sutured to the toads. As such, the control animals also underwent anaesthesia although non-transected control animals did not undergo any sham treatment (a hole

drilled in the skull without transecting the brain) because this is not a very invasive procedure.

Following 9 days of CHC (or control conditions) acute breathing trials were performed within a small (15 cm×15 cm×9 cm) plastic chamber in which the animals were exposed to increasing concentrations of inspired CO_2. Toads were placed into the experimental chamber for 1 h prior to commencing the breathing trials. During this period, the chamber was ventilated (1 l min^{-1}) with room air. The chamber was then gassed with increasing concentrations of CO_2 (2.5%, 3.5%, 4.5% and 5.5%; 20 min per level). These CO_2 levels were achieved by mixing CO_2 with air using Aalborg (Georgetown, NY, USA; model GFC 171) and Sierra (Smart Trak 100) digital mass flow controllers. The levels of CO_2 and O_2 within the experimental chamber were continuously monitored using CO_2 (CD-3A, AEI Technologies) and O_2 (S-3A/I, AEI Technologies) analysers.

Series 5: effects of CHC and olfactory denervation on the hypercapnic ventilatory response

Toads were anaesthetised, as described above, and fitted with impedance leads. In the olfactory-denervated groups, a thin slit was drilled in the skull above the border between the olfactory lobes and the forebrain. A sharp blade was inserted through the hole to cut the olfactory nerves. The hole was then packed with cotton and covered with dental dam. Sham experiments were not performed for reasons described above. The animals were allowed to recover for 48 h before being divided into the following four groups: (1) normocapnic controls (olfactory nerves intact; N=9); (2) normocapnic controls (olfactory denervated; N=6); (3) chronically hypercapnic (olfactory nerves intact; N=8); and (4) chronically hypercapnic (olfactory-denervated; N=7). Each group subsequently underwent acute breathing trials.

Before beginning the acute breathing trials, animals were exposed to room air for 60 min. At the end of this period, the animals were exposed to acute hypercapnia (21% O_2; 5% CO_2) for 20 min and then returned to room air for 20 min. Throughout these trials, the O_2 and CO_2 levels within the chamber were monitored with the O_2 and CO_2 analysers, respectively. Olfactory denervation was confirmed, *post mortem*, by autopsy.

Data analysis

In vitro: in series 1 and 2, the final 10 min of data at each pH level was analysed to determine mean values for fictive breathing frequency (fictive breaths min^{-1}), the number of fictive breaths per episode, the number of fictive episodes per minute and fictive breath duration (s). Fictive breaths in a given episode were defined as occurring within 2 s of each other, according to general practices in the literature (Kinkead et al., 1997; Reid and Milsom, 1998). The area under the integrated (moving average) trace may be taken as an index of fictive breath amplitude. However, given that respiratory-related motor output from the vagus is primarily related to glottal opening and closing [laryngeal branch of the vagus (Sakakibara, 1984a)] rather than buccal pumping [pharyngeal posterior superior branch of the vagus (Sakakibara, 1984a; Sakakibara, 1984b)], integrated vagal activity may not be the ideal index of breath amplitude or tidal volume. A larger breath would, presumably, require that the glottis remain open for a longer period of time.

To further examine whether the output/sensitivity of central respiratory-related chemoreceptors was altered by CHC in series 1 and 2 (prior to transection) the slopes of the pH-fictive breathing dose–response curves (first order regression) were determined for each animal and a mean value was obtained.

In vivo: in series 3 and 4, the final 10–15 min of each experimental period was analysed to determine breathing frequency, breaths per episode, episodes per minute and breath duration. To determine breath amplitude, any DC offset was mathematically subtracted from the impedance trace and the resulting trace was integrated using the DI194 analysis software. The integrated area of the breath was taken as a measure of breath amplitude or tidal volume. The product of breathing frequency and breath amplitude gave an index of total ventilation.

In series 5, values for breathing frequency were determined for the last 10 min of each 20 min period during exposure to room air and 5% CO_2. Following the return to air, this value was determined for the first 5 min following the CO_2 to air transition in order to examine the CO_2-off response (Kinkead and Milsom, 1996). Since the olfactory chemoreceptors affect breathing frequency, other variables were not analysed.

Statistical analysis

In vitro: in all *in vitro* experiments, the values at each pH level in the control and chronically hypercapnic groups, were compared with a one-way repeated measures (RM) analysis of variance (ANOVA) followed by a Dunn's multiple comparison test with a single control point (the highest pH value). In series 1, differences between the control and chronically hypercapnic group were analysed using a two-way ANOVA with pH and control/CHC as the two factors. In series 2, the effects of midbrain transection, CHC and pH were assessed with a three-way ANOVA. In series 1 and 2, the slopes of the dose response curves were compared using a *t*-test (control *versus* CHC).

In vivo: in series 3, the values on each day were compared using a one-way RM ANOVA followed by a Dunn's multiple comparison test. In series 4, the effects of acute hypercapnia were analysed within each group using a one-way RM ANOVA. The effects of CHC and midbrain transection were analysed using a three-way ANOVA. In series 5, the effects of CHC and olfactory denervation were compared using a two-way ANOVA.

All statistical analysis was performed using commercial software (SigmaStat 3.0, Jandel Scientific (SPSS), Chicago, IL, USA). The software determined the appropriate parametric or non-parametric tests as well as the appropriate *post-hoc* multiple comparison test, which followed all analyses of variance. In all cases, the limit of significance was set at 5% (P=0.05).

Results

Series 1 (fictive hypercapnic ventilatory response in vitro)

In the CHC group, fictive breathing frequency (Fig. 1A) increased as aCSF pH was lowered (P=0.029). This was mediated by an increase in the number of fictive episodes per minute (Fig. 1B; P=0.022) rather than fictive breaths per episode (Fig. 1C). There was no significant increase in any of these variables in the control group. The fictive breathing frequency (P=0.001) and the number of episodes per minute (P=0.017) were greater in the CHC toads, than in the control animals. Neither integrated vagal activity (Fig. 1D) nor fictive breath duration (Fig. 1E) were altered by pH or CHC.

A two-way ANOVA revealed no interactive effect between CHC and pH with respect to breathing frequency (P=0.962) and episodes per minute (P=0.662) suggesting that CHC did not alter the sensitivity (gain; slope) of the response to changes in aCSF pH. However, further analysis revealed that the slope of the breathing frequency response curve in Fig. 1A was greater (P=0.028) in the CHC group (slope=–18.8±3.8 fictive breaths min^{-1} pH $unit^{-1}$) than in the control group (–2.2± 4.9 fictive breaths min^{-1} pH $unit^{-1}$).

Series 2 (midbrain transection in vitro)

In this series, consistent with the data in series 1, CHC caused an increase, compared with the control group, in

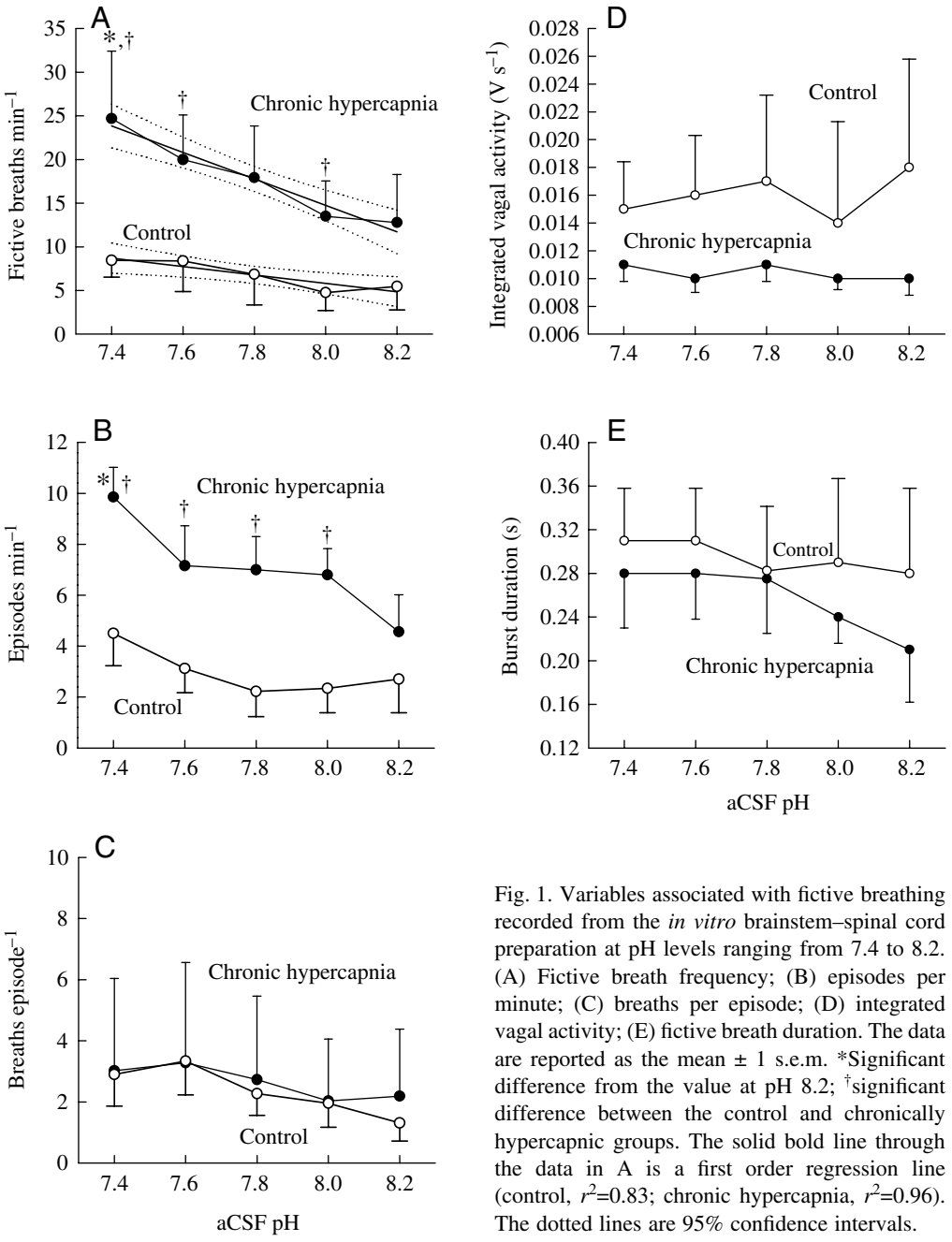

Fig. 1. Variables associated with fictive breathing recorded from the *in vitro* brainstem–spinal cord preparation at pH levels ranging from 7.4 to 8.2. (A) Fictive breath frequency; (B) episodes per minute; (C) breaths per episode; (D) integrated vagal activity; (E) fictive breath duration. The data are reported as the mean ± 1 s.e.m. *Significant difference from the value at pH 8.2; †significant difference between the control and chronically hypercapnic groups. The solid bold line through the data in A is a first order regression line (control, r^2=0.83; chronic hypercapnia, r^2=0.96). The dotted lines are 95% confidence intervals.

fictive breathing frequency at each pH level (Fig. 2A; $P<0.001$). These differences were a result of an increase in the number of fictive episodes per minute (Fig. 2B; $P<0.001$) rather than the number of fictive breaths per episode (Fig. 2C). Midbrain transection had no effect on the frequency response to altered aCSF pH in either the control ($P=0.901$) or CHC ($P=0.803$) groups (Fig. 2A). By contrast, midbrain transection caused a significant increase ($P=0.008$) in the number of fictive episodes per minute in the control group at pH levels of 8.0 and 7.8. This increase did not translate into an increase in fictive breathing frequency because of the non-significant trend for transection to reduce the number of fictive breaths per episode in the control group ($P=0.218$; Fig. 2C).

With respect to fictive breathing frequency and episodes per

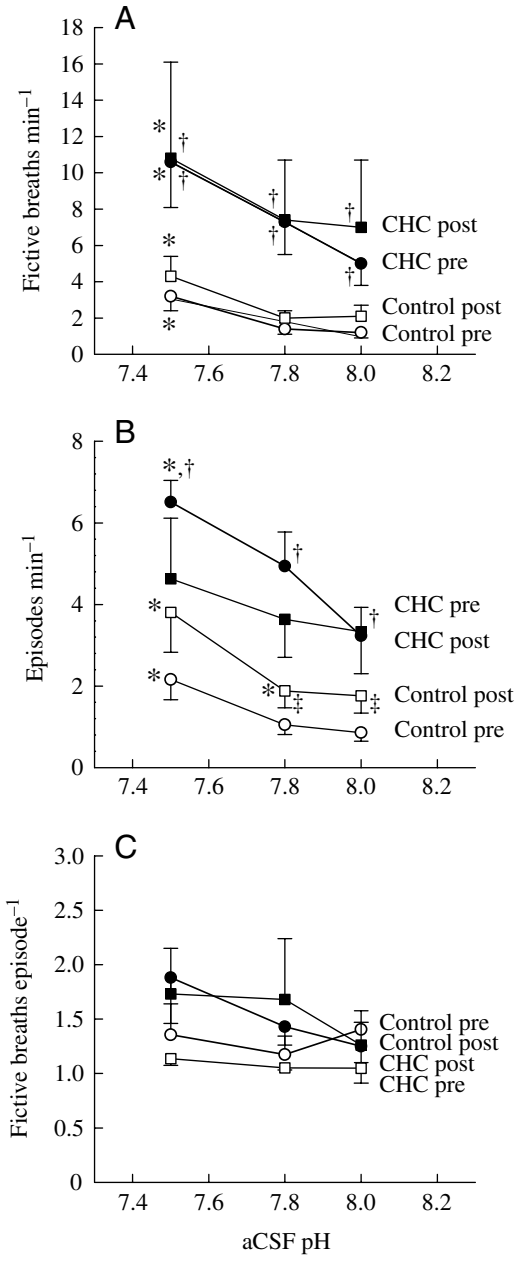

Fig. 2. The effects of midbrain transection on fictive (A) breathing frequency, (B) episodes per minute and (C) breaths per episode recorded *in vitro* from control (open symbols) and chronically hypercapnic (CHC; closed symbols) brainstem–spinal cord preparations. Circles and squares represent values recorded prior to and following midbrain transection, respectively. *Significant difference from the value at pH 8.0 within any given group; †significant difference between the control and CHC groups; ‡significant difference before and after transection in either group.

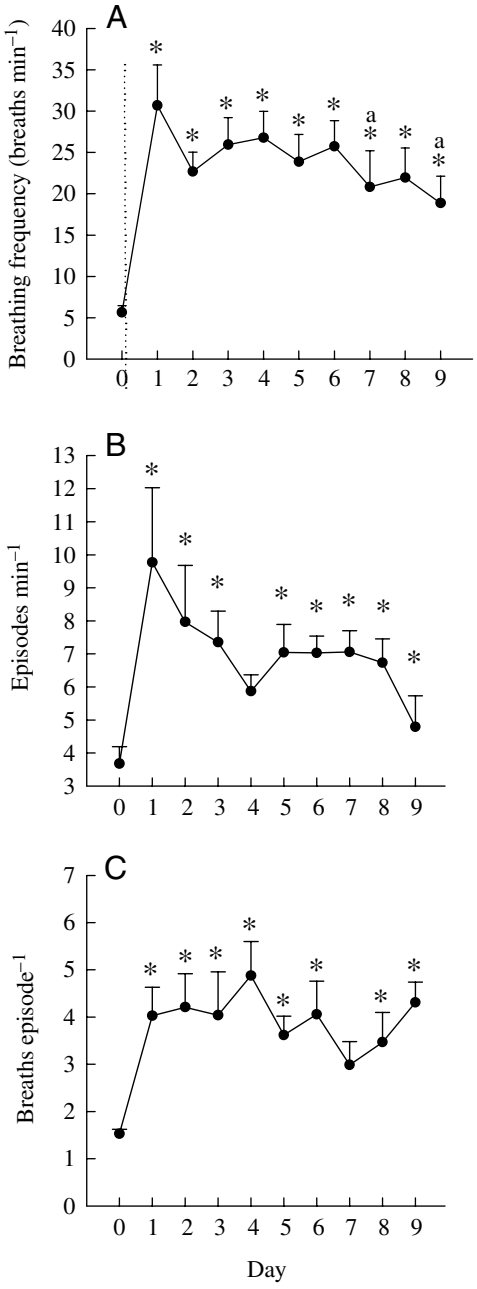

Fig. 3. Variables associated with breathing during a 9 day exposure to CHC *in vivo*. (A) Breathing frequency; (B) episodes per minute; (C) breaths per episode. *Significant difference from the day 0 value. The letter 'a' indicates a significant difference (on days 2–9) from the value on day 1.

minute, the results of the three-way ANOVA did not reveal any interactive effects between CHC, transection and pH. However, further analysis revealed that the slope of the breathing frequency response curve (pre-transection) in Fig. 2A was greater (P=0.016) in the CHC group (slope=–15.1±3.8 fictive breaths min^{-1} pH $unit^{-1}$) than in the control group (–4.2±1.6 fictive breaths min^{-1} pH $unit^{-1}$). There was also a significant (P=0.014; three-way ANOVA) interaction between CHC, pH and transection with respect to fictive episodes per minute.

Series 3 (breathing during the 9 days of CHC)

Fig. 3 illustrates that breathing frequency (Fig. 3A; P<0.001), episodes per minute (Fig. 3B; P=0.008) and

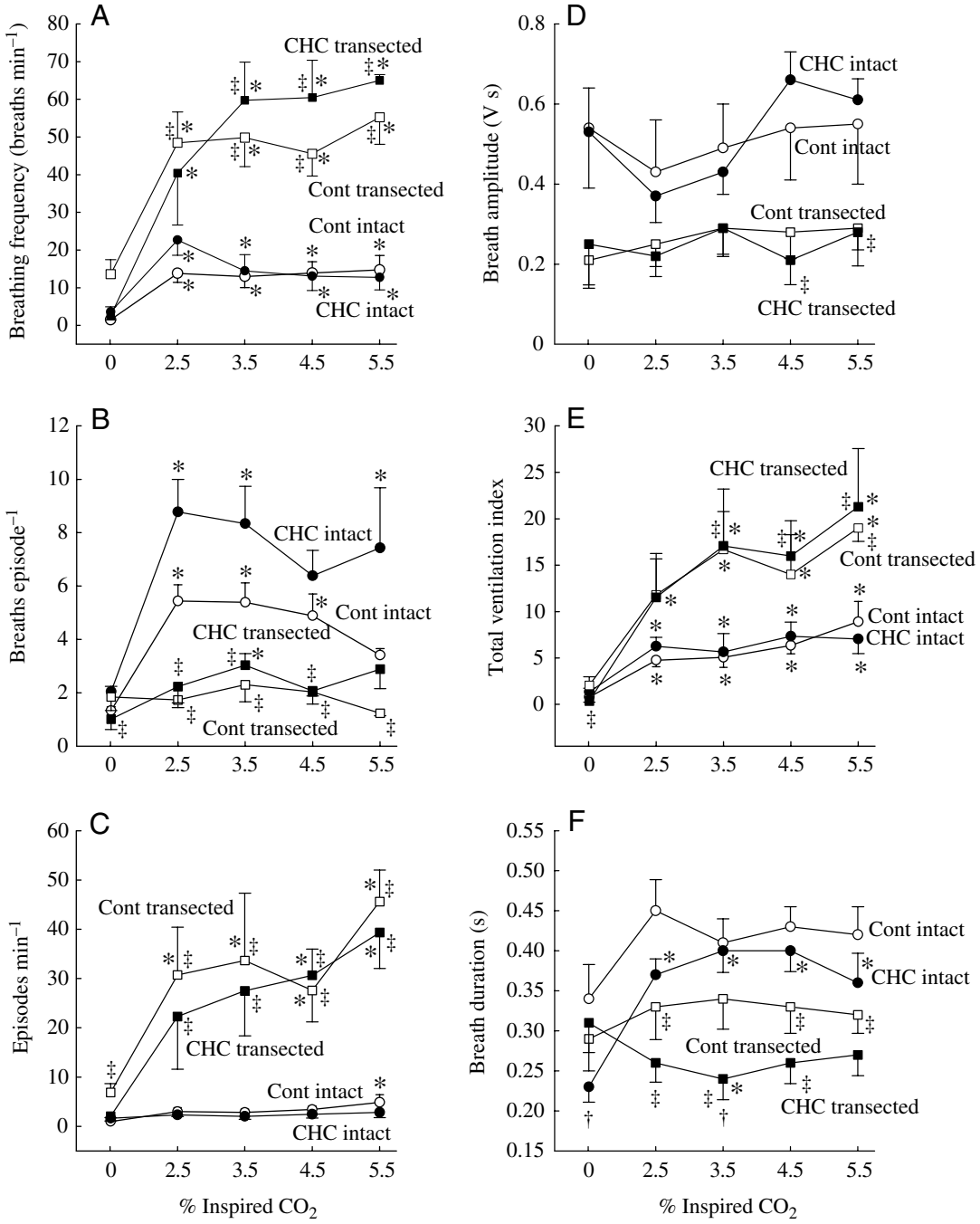

Fig. 4. The effects of CHC and midbrain transection on the acute hypercapnic ventilatory response recorded *in vivo*. Open symbols represent control (Cont) animals; closed symbols represent chronically hypercapnic (CHC) animals. Circles and squares represent values recorded prior to (intact) and following (transected) *in vivo* midbrain transection, respectively. (A) Breathing frequency; (B) breaths per episode; (C) episodes per minute; (D) breath amplitude; (E) total ventilation index; (F) breath duration. *Significant difference from the value at $[CO_2]$=0% within any given group; †significant difference between the control and CHC groups; ‡significant difference before and after transection in either group.

breaths per episode (Fig. 3C; $P<0.001$) were significantly elevated over the 9 day period of CHC compared to day 0 (pre CHC).

Series 4 (hypercapnic ventilatory responses in vivo)

Exposure to acute hypercapnia *in vivo*, with the midbrain intact, caused a significant increase in breathing frequency in both the control ($P<0.001$) and CHC ($P=0.002$) animals (Fig. 4A, asterisks). This was mediated by an increase in the number of breaths per episode (control, $P<0.001$; CHC, $P=0.004$; Fig. 4B) rather than the number of episodes per minute (Fig. 4C). There was no effect of CHC on breathing frequency ($P=0.342$), breaths per episode ($P=0.579$) or episodes per minute ($P=0.218$).

Following transection of the midbrain, acute hypercapnia caused a large increase in breathing frequency (Fig. 4A, asterisks; control and CHC; $P<0.001$) that was mediated by changes in the number of episodes per minute (Fig. 4C, asterisks; control, $P=0.001$; CHC, $P=0.017$). In both the control and CHC groups, midbrain transection caused a significant increase in breathing frequency (Fig. 4A, double daggers; $P<0.001$) and episodes per minute (Fig. 4C, double daggers; $P=0.023$) as well as a significant decrease in the number of breaths per episode (Fig. 4B, double daggers; $P<0.001$). Acute hypercapnia did not alter breath amplitude in either group both before and after midbrain transection (Fig. 4D). In the CHC group, midbrain transection caused a significant decrease in breath amplitude when the animals were breathing 4.5% and 5.5% CO_2 ($P<0.001$).

The total ventilation index (breathing frequency × breath amplitude) increased in all groups in response to acute hypercapnia (Fig. 4E, asterisks; $0.001<P<0.015$). CHC had no effect on total ventilation ($P=0.585$) but midbrain transection caused a significant increase in both groups (Fig. 4E, double daggers; $P<0.001$).

Breath duration increased in the CHC group during exposure to acute hypercapnia before brain transection (Fig. 4F, asterisks; $P<0.001$). This did not occur in the control group ($P=0.096$). Brainstem transection caused a significant decrease in breath duration in both groups (Fig. 4F, double daggers; $P<0.001$).

Series 5 (olfactory denervation in vivo)

Fig. 5A,C illustrates that, during the acute hypercapnic breathing trials, breathing frequency increased during exposure to 5% CO_2 in control and CHC toads ($P<0.001$). Upon return to air (CO_2-off), breathing frequency increased in both groups in an identical manner ($P=0.975$). Following olfactory denervation, breathing frequency increased during exposure to 5% CO_2 in both the control (Fig. 5B) and CHC (Fig. 5D) groups. However, upon return to air (CO_2-off) the breathing frequency in the denervated animals did not change in the control group (Fig. 5B; $P=1.00$) but decreased in the CHC group (Fig. 5D; $P<0.001$).

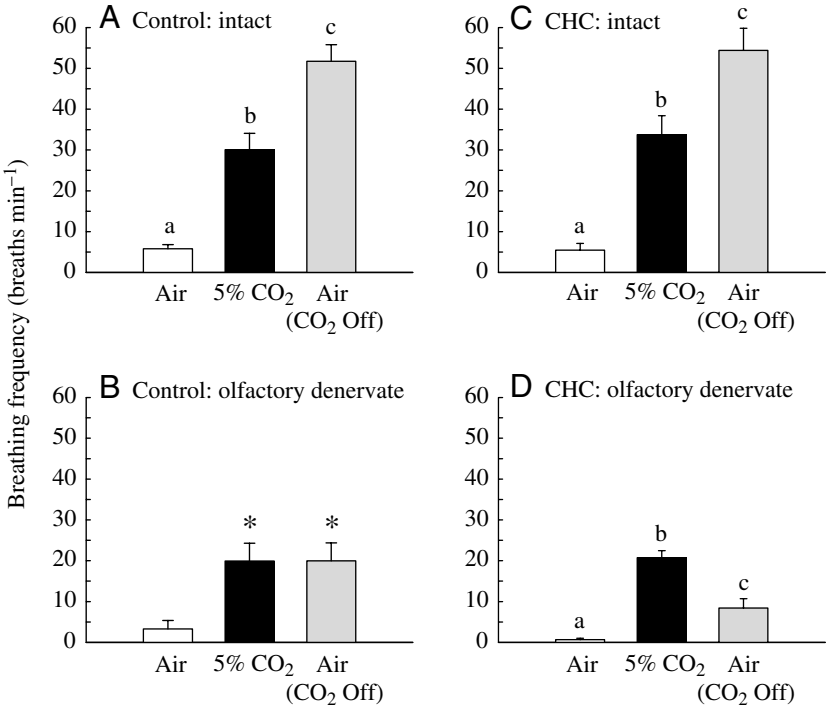

Fig. 5. The effects of chronic hypercapnia (CHC) (C,D) or control normocapnia (A,B) on the breathing frequency response to acute hypercapnia *in vivo* with the olfactory nerves intact (A,C) or denervated (B,D). The letters 'a', 'b' and 'c' indicate that the three values in each panel are significantly different from one another. The asterisk in B indicates a significant difference from the first air value (white bar).

Discussion

Chronic hypercapnia increases central pH/CO_2-sensitive fictive breathing

One of the major results of this study was that central pH/CO_2 chemoreceptor-sensitive fictive breathing was enhanced by CHC. CHC caused an increase in the offset of the response to altered aCSF pH such that the level of activity (fictive breathing frequency) was elevated for any given level of pH. This enhancement was mediated by a change in the number of episodes per minute rather than breaths per episode, suggesting differential regulation or modulation of these two variables following CHC. The different slopes of the pH dose–response curves suggest that CHC may have also caused an increase in the gain (sensitivity) of pH-sensitive fictive breathing to a reduction in aCSF pH. However, this suggestion is tempered by the fact that there was no interactive effect of pH and CHC when the data were compared with a two-way ANOVA.

The increase in central pH/CO_2 chemosensitivity could be explained by a change (increase) in any of the stages in the signal transduction pathway (Lahiri and

Forster, 2003) from the chemoreceptor cell to respiratory motor output. Reid et al. (Reid et al., 2000a) demonstrated, using *in vitro* brainstem–spinal cord preparations from the bullfrog, that a transection slightly caudal to the optic chiasma converted episodic fictive breathing to continuous fictive breathing with a concomitant increase in fictive breathing frequency. A recent study from this laboratory (J. McAneney and S. Reid, unpublished) demonstrated that, in cane toads, 10 days of exposure to chronic normobaric hypoxia (10% O_2) tended to cause a decrease in central pH/CO_2 chemosensitivity measured using the *in vitro* brainstem–spinal cord preparation. Furthermore, a transection caudal to the optic chiasma greatly enhanced the pH/CO_2 chemosensitivity that had been attenuated by chronic hypoxia. Given these previous results, we hypothesised that the increase in central pH/CO_2 chemosensitivity observed in this current study may have resulted from modulation of medullary pH/CO_2 chemoreceptors by midbrain influences.

If CHC caused changes in mechanisms within the midbrain that influence central chemosensitivity, then one could predict that transection of the midbrain would abolish this enhancement. In the current study *in vitro*, the dose response curves in the CHC group were the same before and after midbrain transection (two-way ANOVA). This suggests that descending inputs do not appear to be involved in the CHC-induced increase in the overall magnitude of fictive breathing at each pH level (see below, *in vivo* versus *in vitro* differences for further discussion).

Other possible mechanisms responsible for CHC-induced augmentation of fictive breathing

Another possible explanation for the increase in central pH/CO_2 sensitivity is an increase in the activity or amount of carbonic anhydrase (CA) within the respiratory-related chemoreceptor cells. CA catalyses the hydration of CO_2 to H^+ and HCO_3^- ions and has been shown to be important in H^+/CO_2 sensing in the carotid body glomus cells as well as in the O_2–CO_2 interaction in the carotid body (Iturriaga and Lahiri, 1991). Lipid membranes are generally impermeable to H^+ ions but diffusion of CO_2 into a chemoreceptor cell can cause intracellular acidification. An increase in CA activity may account, at least in part, for the increased central chemosensitivity following CHC.

Another possibility is that altered feedback from lung pulmonary stretch receptors (PSR), during the period of CHC, may have modulated the function of the central CO_2 chemoreceptors. In anurans, pulmonary vagotomy eliminates the acute hypercapnic ventilatory response (Reid et al., 2000b) and electrical stimulation of the pulmonary vagus nerve, *in vitro*, enhances central CO_2 chemosensitivity (Kinkead et al., 1994). PSR feedback, therefore, is important for regulating respiratory-related central pH/CO_2 chemosensitivity. Furthermore, PSR are CO_2 sensitive, with increased levels of CO_2 causing a decrease in receptor activity (see below).

Alternately, given that CHC leads to elevated levels of breathing during the 9 day exposure to elevated CO_2, it is possible that CHC is also associated with elevated arterial P_{O_2} levels. Given that cane toads are discontinuous breathers, arterial gas levels fluctuate depending upon whether or not the animals are breathing, the time taken between breathing episodes and the presence or absence of cardiac shunting. If CHC induces more-or-less continuous breathing (as it appears to do based on Fig. 3A), then arterial P_{O_2} levels would probably be elevated compared to an animal breathing air in a discontinuous manner. Given this, one may hypothesise that reduced levels of afferent input from the carotid labyrinth/aortic arch O_2 chemoreceptors may have altered central pH/CO_2 chemoreceptor function. However, these arterial chemoreceptors are also sensitive to pH/CO_2 so increased afferent input may have occurred during CHC. Regardless, the possibility remains that peripheral chemoreceptor input modified central chemoreceptor function. We are currently investigating the role of carbonic anhydrase, PSR feedback and O_2 chemoreceptor afferent input on central pH/CO_2 chemosensitivity following CHC.

In the control (not chronically hypercapnic) animals, there was no increase in fictive breathing with reduced aCSF pH in series 1 although there was a small but significant increase in series 2. A reduced pH sensitivity *in vitro* is not unexpected given the absence of afferent input to the brainstem–spinal cord preparation (Milsom et al., 1999). Indeed, Morales and Hedrick (Morales and Hedrick, 2002) reported that fictive breathing frequency, recorded from an adult bullfrog brainstem–spinal cord preparation, changed from approximately 1.5 to 3.75 fictive breaths min^{-1} as the pH of the aCSF was lowered from 8.3 to 7.5 at 20°C. The magnitude of this relatively small change is similar to the small change observed in the current study in the control preparations (5.5–8.5 fictive breaths min^{-1} with a pH change from 8.2 to 7.4). Nevertheless, it is without doubt that these preparations are pH sensitive. The observation that central respiratory-related pH sensitivity is reduced *in vitro*, compared to *in vivo*, further suggests that enhanced afferent input during CHC may have altered central pH chemoreceptor function.

In vitro versus in vivo responses

The *in vivo* experiments (series 3) demonstrated that there was a sustained increase, over resting levels (day 0) in breathing frequency during the 9 days of CHC. Given that the central pH/CO_2 chemoreceptors are responsible for the majority of the acute hypercapnic ventilatory response in anuran amphibians (Branco et al., 1992; Smatresk and Smits, 1991), and that central chemoreceptor activity was elevated in the *in vitro* experiments following CHC, it is possible that the sustained increase in breathing frequency *in vivo* was a result of continual activation of these receptors.

Studies on ventilatory responses and blood acid–base changes to hypercapnia in anuran amphibians have typically focused on the time scale of several minutes to 24 h. Toews and Heisler (Toews and Heisler, 1982) demonstrated, in *Bufo marinus*, that 24 h of hypercapnia (5% CO_2) produced a 30% pH compensation in the extracellular fluid and 44%, 65% and

77% compensation in the intracellular compartments of skin, skeletal muscle and cardiac muscle, respectively. These authors concluded that this species preferentially regulates intracellular, over extracellular, pH during this time frame of hypercapnia. Boutilier and Heisler (Boutilier and Heisler, 1988) also demonstrated a relatively limited capacity to compensate extracellular pH (approximately 30% compensation) following 24 h of exposure to 5% CO_2 in *Bufo*. Snyder and Nestler (Snyder and Nestler, 1991) demonstrated that the brain of *Bufo marinus* has a relatively low buffering capacity compared to the liver. It is possible that a poor or comparatively reduced buffering capacity may have contributed to the maintained elevation of breathing during the 9 days of CHC in the current study. A limited buffering capacity in the brain may have facilitated the increased sensitivity of the central pH/CO_2 chemoreceptors. The continual elevation of breathing frequency over the 9 day period suggests the occurrence of a persistent respiratory acidosis. However, breathing frequency had returned to resting levels following 1 h exposure to room air at the beginning of the acute *in vivo* breathing trials (series 4). This would suggest that the *in vivo* hypercapnic ventilatory chemoreflex, that we measured, was not being attenuated by a continual respiratory acidosis, although blood pH and P_{CO_2} measurements would be required to confirm this. The continual elevation of breathing frequency during CHC is consistent with comparable studies on rats [(Lai et al., 1981) 3 weeks of CHC], dogs [(Jennings and Chen, 1976) 14 days of CHC] and humans [(Schaefer et al., 1963) 42 days of CHC]. However, McKenzie et al. (McKenzie et al., 2003) demonstrated that 6 weeks of CHC (water P_{CO_2}=15–45 mmHg) did not result in an elevation of breathing in the European eel (*Anguilla anguilla*).

Given that burrowing animals, exposed to relatively high levels of CO_2, generally have a reduced acute hypercapnic ventilatory response, we hypothesised that CHC would reduce the respiratory response to a subsequent bout of acute hypercapnia in cane toads. Clearly this hypothesis was not supported by either the *in vitro* or *in vivo* experiments. The results indicate that the increase in central pH/CO_2 sensitivity measured *in vitro* does not manifest as an increase in the acute hypercapnic ventilatory response *in vivo*. Furthermore, midbrain transection *in vivo* augmented the acute hypercapnic ventilatory response whereas *in vitro* there was no apparent effect. Given the multiple populations of CO_2-sensitive respiratory-related chemoreceptors in these animals and the central integration of chemoreceptor drive (Kinkead et al., 1997; Gargaglioni and Branco, 2004), this result perhaps is not surprising. *In vivo*, it is possible that the transections (slightly caudal to the optic chiasma) may have influenced the function of the nucleus isthmi which is a midbrain structure that has previously been shown to be important in the hypercapnic ventilatory response in anurans (Kinkead et al., 1997; Gargaglioni and Branco, 2004). Further experiments will examine the role of the nucleus isthmi on central chemosensitivity following CHC by pharmacologically ablating (Kinkead et al., 1997) the nucleus isthmi prior to CHC and *in vitro* experiments.

Brain transection did, however, alter the breathing pattern during acute hypercapnia. With the brains intact, the increase in breathing frequency was mediated by an increase in the number of breaths per episode while the number of episodes per minute remained constant. Following transection, the acute hypercapnic ventilatory response (frequency) was mediated by increases in episodes per minute while breaths per episode did not change. These data are consistent with the differential regulation of these two components of breathing frequency. At the higher levels of inspired CO_2, this is also consistent with breathing becoming more-or-less continuous rather than clustered or episodic.

Olfactory CO_2 chemoreceptors in the nares inhibit breathing frequency. As such, following exposure to acute hypercapnia, there is an immediate increase in breathing frequency following a return to breathing room air. This paradoxical increase in breathing frequency, at a time when the CO_2 drive to breathe is decreasing, results from removal of this inhibitory input (Kinkead and Milsom, 1996; Coates, 2001). Kinkead and Milsom (Kinkead and Milsom, 1996) observed that olfactory denervation in the bullfrog enhanced the increase in breathing frequency during acute hypercapnia (6% CO_2; 1 h). This enhancement was not observed in the current study during acute hypercapnia (5% CO_2; 20 min). The differences may be due to the slightly different experimental protocols.

We hypothesised that the lack of an augmented *in vivo* hypercapnic ventilatory response following CHC may have resulted from increased inhibitory input from these olfactory CO_2 chemoreceptors, which in turn, may have nullified an augmentation of central chemoreceptor function. However, although olfactory denervation appeared to reduce breathing frequency during acute hypercapnia, this effect was observed in both the control and CHC groups, suggesting that CHC did not alter the inhibitory input from these receptors. Changes in these receptors do not appear to cause the *in vitro* versus *in vivo* differences observed in this study.

However, following olfactory denervation in the chronically hypercapnic group, breathing frequency decreased upon return to normocapnic conditions (Fig. 5D). This was not expected, as any respiratory acidosis immediately following acute 5% CO_2 exposure would probably have had a greater stimulatory effect in the chronically hypercapnic group because of the increased sensitivity of the central CO_2 chemoreceptors (had this effect occurred *in vivo*). One possible explanation for the decrease in breathing frequency following a return to room air is a change in the CO_2 sensitivity of lung PSR following CHC. Amphibian PSR are CO_2 sensitive and decrease their firing rate as CO_2 levels are increased. Upon the removal of the inspired 5% CO_2, the [CO_2] in the lung gas would probably have decreased rapidly. This would reduce the inhibitory effects of CO_2 on the PSR and increase their rate of afferent signalling to the brain. In this situation, it is possible that increased amounts of PSR feedback (probably a tonic component) may have caused the decrease in breathing frequency immediately

following acute hypercapnia. Given that this decrease did not occur in the control (i.e. not chronically hypercapnic) group, it is possible that CHC increased the CO_2 sensitivity of lung stretch receptors leading to a greater level of discharge following the removal of CO_2 from the lung gas. If tonic PSR feedback dominated over phasic PSR feedback during this period (Reid and West, 2004) then this could have resulted in a reduced breathing frequency. The absence of PSR feedback in the *in vitro* preparation is a significant difference from the *in vivo* situation. Any effect of PSR feedback on chemosensitivity following CHC is an area that we plan to actively investigate in the near future.

Conclusion

This study demonstrates that exposure to chronic hypercapnia increased the offset of CO_2-sensitive fictive breathing *in vitro* suggesting that CHC altered central CO_2 chemoreceptor function. This response does not appear to have been caused by CHC-induced changes in central descending inputs originating from the midbrain. CHC did not augment the *in vivo* acute hypercapnic ventilatory response whereas midbrain transection enhanced the ventilatory response during acute hypercapnia and the altered breathing pattern. The presence of multiple populations of respiratory-related CO_2-sensitive chemoreceptors in anurans may explain why the increased CO_2 sensitivity observed *in vitro* was not manifest *in vivo*. We are continuing to investigate the effects of olfactory CO_2 chemoreceptors, PSR and arterial O_2/CO_2 chemoreceptors on central chemosensitivity.

This study was supported by NSERC of Canada Discovery and Equipment grants to S.G.R. S.G.R. is currently supported by a Parker B. Francis Fellowship in Pulmonary Research (Francis Families Foundation). Funding for equipment was also provided by the Canadian Foundation for Innovation, the Ontario Innovation Trust and the Ontario Thoracic Society. A.G. was the recipient of NSERC undergraduate summer scholarships (2003 and 2004) and currently holds an Ontario Graduate Scholarship.

References

Boggs, D. F., Kilgore, D. L., Jr and Birchard, G. F. (1984). Respiratory physiology of burrowing mammals and birds. *Comp. Biochem. Physiol.* **77A**, 1-7.

Boutilier, R. G. and Heisler, N. (1988). Acid–base regulation and blood gases in the anuran amphibian, *Bufo marinus*, during environmental hypercapnia. *J. Exp. Biol.* **134**, 79-98.

Boutilier, R. G., Randall, D. J., Shelton, G. and Toews, P. (1979a). Acid–base relationships in the blood of the toad, *Bufo marinus*. I. The effects of environmental CO_2. *J. Exp. Biol.* **82**, 331-344.

Boutilier, R. G., Randall, D. J., Shelton, G. and Toews, D. P. (1979b). Acid–base relationships in the blood of the toad, *Bufo marinus*. III. The effects of burrowing. *J. Exp. Biol.* **82**, 357-365.

Branco, L. G., Glass, M. L. and Hoffman, A. (1992). Central chemoreceptor drive to breathing in unanaesthetised toads, *Bufo paracnemis*. *Respir. Physiol.* **87**, 195-204.

Breckenridge, W. J. and Tester, J. R. (1961). Growth, local movements and hibernation of the Manitoba toad, *Bufo hemiophrys*. *Ecology* **42**, 637-646.

Coates, E. L. (2001). Olfactory CO_2 chemoreceptors. *Respir. Physiol.* **129**, 219-229.

Gargaglioni, L. H. and Branco, L. G. (2004). Nucleus isthmi and control of breathing in amphibians. *Respir. Physiol. Neurobiol.* **143**, 177-186.

Iturriaga, R. and Lahiri, S. (1991). Carotid body chemoreception in the absence and presence of CO_2-HCO_3^-. *Brain Res.* **568**, 253-260.

Jennings, D. B. and Chen, C. C. (1976). Ventilation in conscious dogs during acute and chronic hypercapnia. *J. Appl. Physiol.* **41**, 839-847.

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., Filmyer, W. G., Mitchell, G. S. and Milsom, W. K. (1994). Vagal input enhances responsiveness of respiratory discharge to central changes in pH/CO_2 in bullfrogs. *J. Appl. Physiol.* **77**, 2048-2051.

Kinkead, R., Harris, M. B. and Milsom, W. K. (1997). The role of the nucleus isthmi in respiratory pattern formation in bullfrogs. *J. Exp. Biol.* **200**, 1781-1793.

Kuhlman, W. D. and Fedde, M. R. (1979). Intrapulmonary receptors in the bullfrog: Sensitivity to CO_2. *J. Comp. Physiol.* **132**, 69-75.

Kuhnen, G. (1986). O_2 and CO_2 concentrations in burrows of euthermic and hibernating golden hamsters. *Comp. Biochem. Physiol.* **84A**, 517-522.

Lahiri, S. and Forster, R. E., II (2003). CO_2/H^+ sensing: peripheral and central chemoreception. *Int. J. Biochem. Cell Biol.* **35**, 1413-1435.

Lai, Y. L., Lamn, J. E. and Hildebrandt, J. (1981). Ventilation during prolonged hypercapnia in the rat. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **51**, 78-83.

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.

McAneney, J. A., Gheshmy, S., Uthayalingam, S. and Reid, S. G. (in press). The effects of chronic hypoxia on NMDA-mediated regulation of the hypoxic ventilatory response in an amphibian, *Bufo marinus. Respir. Physiol. Neurobiol.*

McKenzie, D. J., Piccolella, M., Dalla Valle, A. Z., Taylor, E. W., Bolis, C. L. and Steffensen, J. F. (2003). Tolerance of chronic hypercapnia by the European eel *Anguilla anguilla*. *J. Exp. Biol.* **206**, 1717-1726.

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

Milsom, W. K. (2002). Phylogeny of CO_2/H^+ chemoreception in vertebrates. *Respir. Physiol. Neurobiol.* **131**, 29-41.

Milsom, W. K. and Jones, D. R. (1977). Carbon dioxide sensitivity of pulmonary receptors in the frog. *Experientia* **33**, 1167-1168.

Milsom, W. K., Reid, S. G., Meier, J. T. and Kinkead, R. (1999). Central respiratory pattern generation in the bullfrog, *Rana catesbeiana*. *Comp. Biochem. Physiol.* **124A**, 253-264.

Morales, R. D. and Hedrick, M. S. (2002). Temperature and pH/CO_2 modulate respiratory activity in the isolated brainstem of the bullfrog (*Rana catesbeiana*). *Comp. Biochem. Physiol.* **132A**, 477-487.

Pinder, A. W., Storey, K. B. and Ultsch, G. R. (1992). Estivation and hibernation. In *Environmental Physiology of the Amphibia* (ed. M. E. Feder and W. W. Burggren), pp. 250-274. Chicago: University of Chicago Press.

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. and West, N. H. (2004). Regulation of episodic breathing by phasic pulmonary stretch receptor feedback in the Cane Toad, *Bufo marinus*. *Respir. Physiol. Neurobiol.* **142**, 165-183.

Reid, S. G., Meier, J. T. and Milsom, W. K. (2000a). The influence of central descending inputs on breathing pattern in the *in vitro* isolated bullfrog (*Rana catesbeiana*) brainstem-spinal cord. *Respir. Physiol.* **120**, 197-211.

Reid, S. G., Milsom, W. K., Meier, J. T., Munns, S. and West, N. H. (2000b). The role of pulmonary stretch receptor feedback in cardiorespiratory control in the Cane toad (*Bufo marinus*). *Respir. Physiol.* **120**, 213-230.

Russell, A. P. and Bauer, A. M. (1993). *The Amphibians and Reptiles of Alberta.* Edmonton: University of Alberta Press.

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

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

Saunders, C. E. and Milsom, W. K. (2001). The effects of tonic lung inflation

on ventilation in the American Bullfrog, *Rana catesbeiana* Shaw. *J. Exp. Biol.* **204**, 2647-2656.

Schaefer, K. E., Hastings, B. J., Carey, C. R. and Nichols, G., Jr (1963). Respiratory acclimatization to carbon dioxide. *J. Appl. Physiol.* **18**, 1071-1078.

Smatresk, N. and Smits, A. W. (1991). Effects of central and peripheral chemoreceptor stimulation on ventilation in the marine toad, *Bufo marinus*. *Respir. Physiol.* **83**, 223-238.

Snyder, G. K. and Nestler, J. R. (1991). Intracellular pH in the toad *Bufo marinus* following hypercapnia. *J. Exp. Biol.* **161**, 415-422.

Taylor, B. E., Harris, M. B., Coates, E. L., Gdovin, M. J. and Leiter, J. C. (2003a). Central CO_2 chemoreception in developing bullfrogs: anomalous response to acetazolamide. *J. Appl. Physiol.* **94**, 1204-1212.

Taylor, B. E., Harris, M. B., Leiter, J. C. and Gdovin, M. J. (2003b). Ontogeny of central CO_2 chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. *Am. J. Physiol.* **285**, R1461-R1472.

Toews, D. P. and Macintyre, D. (1978). Respiration and circulation in an apodan amphibian. *Can. J. Zool.* **56**, 998-1004.

Toews, D. P. and Heisler, N. (1982). The effects of hypercapnia on intracellular and extracellular acid-base status in the toad *Bufo marinus*. *J. Exp. Biol.* **97**, 79-86.

Van Vliet, B. N. and West, N. H. (1992). Functional characteristics of arterial chemoreceptors in an amphibian (*Bufo marinus*). *Respir. Physiol.* **88**, 113-127.

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

Wang, T., Taylor, T., Reid, S. G. and Milsom, W. K. (2004). Interactive effects of mechano- and chemo-receptor inputs on cardio-respiratory outputs in the toad. *Respir. Physiol. Neurobiol.* **140**, 63-76.

West, N. H., Topor, Z. L. and Van Vliet, B. N. (1987). Hypoxemic threshold for lung ventilation in the toad. *Respir. Physiol.* **70**, 377-390.