The Journal of Experimental Biology 210, 227-237
Published by The Company of Biologists 2007
doi:10.1242/jeb.02606

Afferent input modulates the chronic hypercapnia-induced increase in respiratory-related central pH/CO_2 chemosensitivity in the cane toad (*Bufo marinus*)

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Accepted 17 October 2006

Summary

The goal of this study was to examine the role of respiratory-related afferent input on the chronic hypercapnia (CHC)-induced increase in central respiratory-related pH/CO_2 chemosensitivity in cane toads (*Bufo marinus*). Toads were exposed to CHC (3.5% CO_2) for 10 days, following which *in vitro* brainstem–spinal cord preparations were used to assess central respiratory-related pH/CO_2 chemosensitivity. Motor output from the vagus nerve root was used as an index of breathing (fictive breathing). Olfactory denervation (OD), prior to exposure to CHC, was used to remove the influence of CO_2-sensitive olfactory chemoreceptors, which inhibit breathing. Exposure to chronic hyperoxic hypercapnia (CHH) was used to reduce the level of arterial chemoreceptor input compared with CHC alone. *In vivo* experiments examined the effects of CHC, CHH and OD on the acute hypercapnic ventilatory response of intact animals. *In vitro*, a reduction in artifical cerebral spinal fluid (aCSF) pH increased fictive breathing in preparations taken from control and CHC animals. CHC caused an increase in fictive breathing compared with controls. OD and CHH abolished the CHC-induced augmentation of fictive breathing. *In vivo*, CHC did not cause an augmentation of the acute hypercapnic ventilatory response. CHH reduced the *in vivo* acute hypercapnic ventilatory response compared with animals exposed to CHC. *In vivo*, OD reduced breathing frequency and increased breath amplitude in both control and CHC animals. The results suggest that afferent input from olfactory and arterial chemoreceptors, during CHC, is involved in triggering the CHC-induced increase in central respiratory-related pH/CO_2 chemosensitivity.

Key words: brainstem–spinal cord preparations, central pH/CO_2 chemoreceptors, chronic hypercapnia, chronic hyperoxia, olfactory chemoreceptors.

Introduction

Although the respiratory responses of anuran amphibians (frogs and toads) to acute hypoxia and hypercapnia have been well documented (Shelton et al., 1986; Milsom, 1991), the effects of chronic hypoxia and hypercapnia on breathing and respiratory control systems have received little attention. Recently, using an *in vitro* brainstem–spinal cord preparation, we demonstrated that exposure of cane toads (*Bufo marinus*, Linnaeus) to 10 days of chronic hypercapnia (CHC; 3.5% CO_2) caused an increase in the magnitude and sensitivity of pH/CO_2-sensitive fictive breathing (Gheshmy et al., 2006). The focus of the current study was to examine the effect of removing afferent respiratory-related input on the CHC-induced increase in respiratory-related central pH/CO_2 chemosensitivity.

There are a multitude of CO_2-sensitive respiratory control systems that regulate anuran breathing (Milsom, 1991; Milsom, 2002). Central pH/CO_2 chemoreceptors, located on the ventrolateral surface of the medulla, trigger increases in breathing when they are stimulated by reductions in cerebral spinal fluid (CSF) pH or increases in CSF CO_2 levels (Smatresk and Smits, 1991; Milsom, 2002). Arterial chemoreceptors, located in the carotid labyrinth and aortic arch, are both O_2- and CO_2-sensitive. Stimulation of these chemoreceptors during acute hypoxia and hypercapnia leads to an increase in breathing (West et al., 1987; Smatresk and Smits, 1991). CO_2-sensitive olfactory chemoreceptors, located in the nasal epithelium (Coates and Ballam, 1990; Coates, 2001), inhibit breathing when stimulated by physiologically relevant levels of inspired CO_2 (Sakakibara, 1978; Coates, 2001; Kinkead and Milsom, 1996; Milsom et al., 2004). Pulmonary stretch receptors (PSR), located in the walls of the lungs, monitor both the breath-by-breath changes in lung volume during inspiration and expiration as well as the degree of lung inflation during periods of apnea. 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).

This study addressed two specific hypotheses regarding the potential for respiratory-related afferent input to trigger or induce the CHC-induced increase in central pH/CO_2 chemosensitivity previously reported by Gheshmy et al. (Gheshmy et al., 2006). First, we hypothesised that the increase in central pH/CO_2 chemosensitivity occurred in order to counter (or offset) enhanced input from CO_2-sensitive olfactory chemoreceptors, which would presumably occur during CHC. An increase in olfactory chemoreceptor input would, theoretically and paradoxically, tend to reduce breathing during a period of elevated respiratory drive (CHC). Second, we hypothesised that the increase in central pH/CO_2 chemosensitivity during CHC was because of increased input from the CO_2-sensitive arterial chemoreceptors located in the carotid labyrinth and aortic arch, which would also be constantly stimulated during CHC. Takakura et al. demonstrated that central chemoreceptor cells in rats can be activated by input from carotid body chemoreceptors (Takakura et al., 2006).

In this study, cane toads were exposed to CHC (3.5% CO_2) for 10 days. The respiratory physiology of this species has been well studied and these animals can encounter chronic hypercapnia in their natural environment (Boutilier et al., 1979a; Toews and Macintyre, 1978). *In vitro* brainstem–spinal cord preparations, in which vagal motor output was used as an index of breathing (fictive breathing), were used to assess respiratory-related central pH/CO_2 chemosensitivity. To address the first hypothesis, the olfactory nerves were transected to eliminate olfactory chemoreceptor input to the brain during the period of CHC. To address the second hypothesis, animals were simultaneously exposed to CHC and chronic hyperoxia (CHO) (chronic hyperoxic hypercapnia; CHH) in order to reduce arterial chemoreceptor input to the brain (compared with CHC alone). The rationale behind this approach was that the arterial chemoreceptor-induced hypercapnic ventilatory response is influenced by arterial O_2 levels (West et al., 1987) and that an elevation in arterial O_2 reduces the CO_2-induced increase in arterial chemoreceptor discharge (Van Vliet and West, 1992). Additional experiments examined whether olfactory denervation (OD) and CHH altered breathing and the acute hypercapnic ventilatory response *in vivo*.

Materials and methods

Experimental animals

Cane toads (*Bufo marinus*) (N=92; 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 had access to 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 L:D. 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 CHC, CHO and CHH

During exposure to CHC, toads were placed, for a 10-day period, in a Plexiglas chamber (35 cm×25 cm×10 cm) within which the inspired CO_2 level was maintained at 3.5±0.1% using a Pro-CO_2 control unit (Biospherix, Redfield, 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 (Gheshmy et al., 2006). Routine measurements of O_2 (S-3A O_2 analyser; AEI Technologies, Pittsburgh, PA, USA) confirmed that inspired O_2 levels remained at approximately 20.2±0.1%. The chamber was maintained at room temperature and exposed to a 12 h:12 h L:D cycle.

CHO was achieved using a Pro-Ox 110 control unit (Biospherix). An O_2 electrode within the chamber monitored the level of O_2. When the O_2 level fell below 30%, the Pro-Ox delivered a small amount of O_2 to raise the level back to 30%. Routine measurements of CO_2 (CD-3A CO_2 analyser; AEI Technologies) confirmed that inspired CO_2 levels remained at approximately 0.03%. CHH conditions (30% O_2 and 3.5% CO_2) were established using the Pro-Ox 110 control unit in conjunction with the Pro-CO_2 control unit.

Olfactory denervation

OD was performed on animals used for *in vitro* brainstem–spinal cord preparation experiments and for *in vivo* acute hypercapnic breathing trial experiments (see below). Toads were anaesthetised by emersion in a solution of 3-aminobenzoic acid ethyl ester (MS222, 1.0 g l^{-1}; Sigma) buffered to pH 7.0 with sodium bicarbonate (Reid and Milsom, 1998; Reid et al., 2000a; Reid et al., 2000b; Gheshmy et al., 2006). Animals were kept in the anaesthetic until the eye-blink and toe-pinch reflexes were eliminated. Using a Dremmel Tool, a thin horizontal slit was drilled above the border between the olfactory lobes and the forebrain. A sharp blade was inserted through the hole and the olfactory nerves were severed. The opening was packed with cotton and covered with dental dam. Animals were allowed to recover for 24 h before being divided into control and CHC groups. Control animals were maintained in room air for a 10-day period. The CHC groups were maintained as described above. OD was confirmed post-mortem.

The surgical procedure used to open the skull in order to perform OD was very minor. In the *in vivo* experiments (see below), OD was performed at the same time as impedance leads were sutured to the toads. As such, control animals also underwent anaesthesia, although there was no sham treatment *per se* for the OD. Given that drilling a thin hole in the skull is not a very invasive procedure, sham experiments (hole drilled in the skull without OD) were not performed (Gheshmy et al.,

2006). Kinkead and Milsom reported no effect, on any variable associated with breathing, of a sham operation for OD (Kinkead and Milsom, 1996).

In vitro experiments

The in vitro brainstem–spinal cord preparation

Animals were anaesthetised as described above. Using a Dremmel Tool, an incision was made in the skull rostral to the optic lobes. The cranial case was removed with Rongeurs and bone shears and placed into a Sylgard-coated dissecting dish. The brain was exposed and superfused with ice-cold oxygenated artificial cerebral spinal fluid (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; Sigma) (Taylor et al., 2003a; Taylor et al., 2003b; Gheshmy et al., 2006). The rostral forebrain was removed and the remaining brain tissue was continually superfused with the aCSF. The transection to remove the rostral forebrain was performed in the middle of the cerebral hemispheres. Based on the Hoffman atlas of the toad brain (Hoffman, 1973), this transection site would correspond to approximately 1.5 mm anterior to the zero mark (defined as the vertex of the angle formed at the point where the cerebral hemispheres become separate or begin to diverge). Internally, this zero point (Hoffman, 1973) corresponds to the interventricular foramen (foramen Monroi). The forebrain transection goes through the primordial hippocampus, the primordial general pallium, the primordial piriform cortex, the lateral septal nucleus, the striatum, the medial forebrain bundle, the medial septal nucleus and the lateral ventricle.

Cranial nerves were cut close to their exit from the skull and the spinal cord was severed at the level of the third spinal nerve. The preparation was transferred from the braincase and immobilised with insect pins in a Sylgard-coated dissecting dish continually superfused with oxygenated aCSF. The dura matter surrounding the brain was 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 (Kinkead et al., 1994; McLean et al., 1995a; McLean et al., 1995b; Gheshmy et al., 2006). 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 (Gheshmy et al., 2006). The preparations were maintained at pH 7.8 and room temperature for 1 h before commencing the experiment.

Suction electrodes of various diameters were made from thin-walled capillary glass (1.2 mm outer diameter, 0.58 mm inner diameter) pulled to a fine tip using a vertical pipette puller (model 720; David Kopf Instruments, 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 aspirated 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 (Fig. 1; see the data analyses section for further details).

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; CWE Inc., Ardmore, PA, USA) for integration (time constant=200 ms) and to an audio monitor (model 3300; AM Systems, Carlsborg, WA, USA). The amplified/filtered nerve signal and integrated signal were monitored and stored using a data acquisition system (MP150; Biopac Systems, 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 (VWR) placed within the aCSF reservoir.

Experimental protocol (in vitro)

Following the 1 h stabilisation period (see above) and the observation of stable levels of fictive breathing, each preparation was exposed to varying levels of aCSF pH (7.5, hypercapnic; 7.8, normocapnic; 8.0, hypocapnic; random order exposure). This pH range approximates that used in previous studies on amphibian brainstem–spinal cord preparations (Kinkead et al., 1994; McLean et al., 1995a; McLean et al., 1995b; Gheshmy et al., 2006). All experiments were performed at room temperature (approximately 22°C). This is within the temperature range (15–25°C) reported by Morales and Hedrick (Morales and Hedrick, 2002), in which fictive breathing is consistently active from *in vitro* adult bullfrog brainstem–spinal cord preparations. Each pH change was achieved over a period of 20 min. Preparations were allowed to acclimatise to each new pH level for a further 20 min before fictive breathing was monitored for an additional 20 min data-collection period.

In vitro experiments were performed on the following groups: (1) control animals (maintained in room air) with the olfactory nerves intact (controls OI; *N*=12); (2) control animals with olfactory denervation (controls OD; *N*=10); (3) chronically hypercapnic animals with the olfactory nerves intact (CHC OI; *N*=12); (4) chronically hypercapnic animals with olfactory denervation (CHC OD; *N*=9); (5) chronically hyperoxic (CHO) animals (OI; *N*=8); and (6) chronically hyperoxic hypercapnic (CHH) animals (OI; *N*=10).

In vivo experiments

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. Previous experiments from this laboratory have validated impedance measurements as an appropriate measure of breathing in this species (McAneney et al., 2006). Impedance measurements made *in vivo* do scale with breath volume (McAneney et al., 2006), although impedance measurements *per se* do not provide a direct quantification of breath volume. Following placement of the impedance leads, animals were allowed to recover from anaesthesia for 48 h prior to the initiation of chronic experimental or control conditions.

In vivo experiments were performed on the following five groups of animals: (1) controls OI (N=7); (2) controls OD (N=6); (3) CHC OI (N=6); (4) CHC OD (N=6); and (5) CHH OI (N=6). These experiments were performed on different animals to those used in the *in vitro* experiments.

Following the 10 days of chronic experimental 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 (normocapnic conditions). The chamber was then gassed (1 l min^{-1}) with increasing concentrations of CO_2 (hypercapnic gas mixtures; 2%, 3% and 4.5%; 30 min per level). These CO_2 levels were achieved by mixing CO_2 with air using Aalborg (model GFC 171; Orangeburg, NY, USA) and Sierra (Smart Trak 100; Sierra Instruments) digital mass flow controllers. Following exposure to each CO_2 level, the chamber was ventilated with room air for 30 min. The levels of CO_2 and O_2 within the experimental chamber were continuously monitored using a CO_2 (CD-3A; AEI Technologies) and O_2 analyser (S-3A/I; AEI Technologies), respectively.

To measure breathing, the impedance leads were connected to extensions that, in turn, input into an impedance converter (model 2991; UFI, Morro Bay, CA, USA). 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.

Data and statistical analyses

In vitro, the final 20 min of data at each pH level were 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 min^{-1}, fictive breath duration (s) and integrated fictive breath area. To determine integrated fictive breath area, we selected 20 fictive breaths during each 20 min data-collection period and determined the integrated area for each of these breaths. These were then used to generate a single value (mean of all the breaths measured) for that particular condition in each preparation. That value was then used to calculate the overall mean value that is reported in the graphs.

In this study we were recording from the root of the whole vagus nerve. Respiratory-related motor activity in this nerve, *in vivo*, controls the opening and closing of the glottis (*via* the laryngeal branch of the vagus) as well as the buccal respiratory pump muscles (*via* the pharyngeal posterior superior nerve) (Sakakibara, 1984a). Since these preparations are devoid of any afferent input and breathing is an inherently rhythmic process generated in the brainstem, all rhythmic (Reid and Milsom, 1998) activity (*in vitro* motor output) recorded from the vagus nerve was assumed to represent motor output to the respiratory muscles.

Sakakibara demonstrated that an increase in buccal pressure was positively correlated with integrated trigeminal nerve activity (r=0.97) (Sakakibara, 1984b). The trigeminal nerve (mandibular branch) innervates buccal pump muscles. However, integrated nerve activity from the pharyngeal posterior superior nerve (vagal branch) is also positively correlated with integrated trigeminal nerve activity (r=0.95) (Sakakibara, 1984b). This suggests that nerve activity in the pharyngeal posterior superior nerve should also be correlated with buccal pressure changes (or breath volume). Furthermore, given that the laryngeal branch of the vagus nerve controls the opening and closing of the glottis, it is possible that a larger volume breath requires that the glottis remains open for a longer period of time compared with a smaller volume breath. Given this, it is likely that a larger breath would be associated with a larger amount (i.e. integrated area) of vagal activity. Based on the above observations (Sakakibara, 1984a; Sakakibara, 1984b) and assumptions, we believe that it is reasonable to assume that the integrated area of vagal activity is a valid index of breath volume.

The total fictive ventilation index was calculated as the product of fictive breathing frequency and integrated fictive breath area. 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., 1994; Kinkead et al., 1997; Reid et al., 2000a; Gheshmy et al., 2006).

In vivo, the final 15 min of each experimental period was analysed to determine breathing frequency (breaths min^{-1}) and the integrated area of the breaths. To determine the integrated area of the breaths, any DC offset was mathematically subtracted from the impedance trace and the resulting trace was integrated using the DI194 analysis software. The product of breathing frequency and the integrated area of the breaths was considered to be an index of total ventilation.

All statistical analyses were performed using commercial software (SigmaStat 3.0; SPSS, Chicago, IL, USA). The plotted data values represent the mean ± 1 standard error of the mean (s.e.m.). *In vitro*, the effects of CHC, OD and aCSF pH were analysed using a three-way analysis of variance (ANOVA) (Figs 2, 3). *In vitro*, the effects of chronic exposure (CHC, CHH, CHO or control conditions) and aCSF pH were analysed with a two-way ANOVA (Fig. 4).

In vivo, the effects of CHC, OD and the inspired CO_2 level (2–4.5% CO_2 breathing trials) were analysed using a three-way ANOVA (Fig. 5). *In vivo*, the effects of CHC and CHH and the acute inspired CO_2 level were determined by a two-way ANOVA (Fig. 6). In all cases (*in vitro* and *in vivo*), each significant ANOVA was followed by a student–Newman–Keuls multiple comparison test. The limit of significance was 5% (P<0.05).

Results

In vitro experiments

Fig. 1 illustrates fictive breathing recorded at an aCSF pH level of 7.5 in brainstem–spinal cord preparations taken from the control (Fig. 1A,B) and CHC (Fig. 1C,D) groups with the olfactory nerves intact (Fig. 1A,C) and following OD (Fig. 1B,D). CHC increased fictive breathing frequency prior to OD (compare Fig. 1A with Fig. 1C). OD had no effect on fictive breathing frequency in the control preparations (compare Fig. 1A with Fig. 1B). Following CHC, OD reduced fictive breathing frequency and eliminated the clustering of fictive breaths into episodes (compare Fig. 1C with Fig. 1D).

The effects of altered aCSF pH in preparations taken from control-OI toads (in vitro)

In preparations taken from control animals with the olfactory nerves intact (control OI; Figs 2, 3, panels A,C,E), a reduction in aCSF pH led to an increase in fictive breathing frequency (Fig. 2A; $P<0.001$) that was mediated by an increase in the number of fictive episodes min^{-1} (Fig. 2C; $P=0.007$) with no change in the number of fictive breaths per episode (Fig. 2E; $P=0.814$). Decreasing aCSF pH did not increase the integrated area of the fictive breaths (Fig. 3A; $P=0.269$) or fictive breath duration (Fig. 3E; $P=0.959$). The total fictive ventilation index increased, slightly but significantly, as aCSF pH was lowered (Fig. 3C; $P=0.006$).

The effects of altered aCSF pH in preparations taken from CHC-OI toads (in vitro)

In preparations taken from animals exposed to CHC with the olfactory nerves intact (CHC OI; Figs 2, 3, panels B,D,F), a reduction in aCSF pH led to increases in fictive breathing frequency (Fig. 2B; $P=0.029$), the number of fictive episodes min^{-1} (Fig. 2D; $P=0.011$) and the number of fictive breaths per episode (Fig. 2F; $P=0.016$). A reduction in aCSF pH had no effect on the integrated area of the fictive breaths (Fig. 3B; $P=0.584$) or fictive breath duration (Fig. 3F; $P=0.371$). The total fictive ventilation index increased as aCSF pH was reduced (Fig. 3D; $P<0.001$).

The effects of CHC in OI animals (in vitro)

Exposure to CHC (in OI animals) caused an increase in fictive breathing frequency at all aCSF pH levels (compare the open symbols in Fig. 2A,B; $P<0.001$). This was mediated by a CHC-induced increase in the number of fictive episodes min^{-1} (compare the open symbols in Fig. 2C with Fig. 2D; $P<0.001$) with no change in the number of fictive breaths per episode (compare the open symbols in Fig. 2E with Fig. 2F; $P=0.327$). CHC had no effect on the integrated area of the fictive breaths (compare the open symbols in Fig. 3A with Fig. 3B; $P=0.116$) or fictive burst duration (compare the open symbols in Fig. 3E with Fig. 3F; $P=0.683$). The total fictive ventilation index was greater in the CHC preparations compared with the control preparations (compare the open symbols in Fig. 3C with Fig. 3D; $P<0.001$).

The effects of OD (in vitro)

With the exception of an increase in fictive breath duration at pH 8.0 (Fig. 3E; $P=0.014$), OD had no

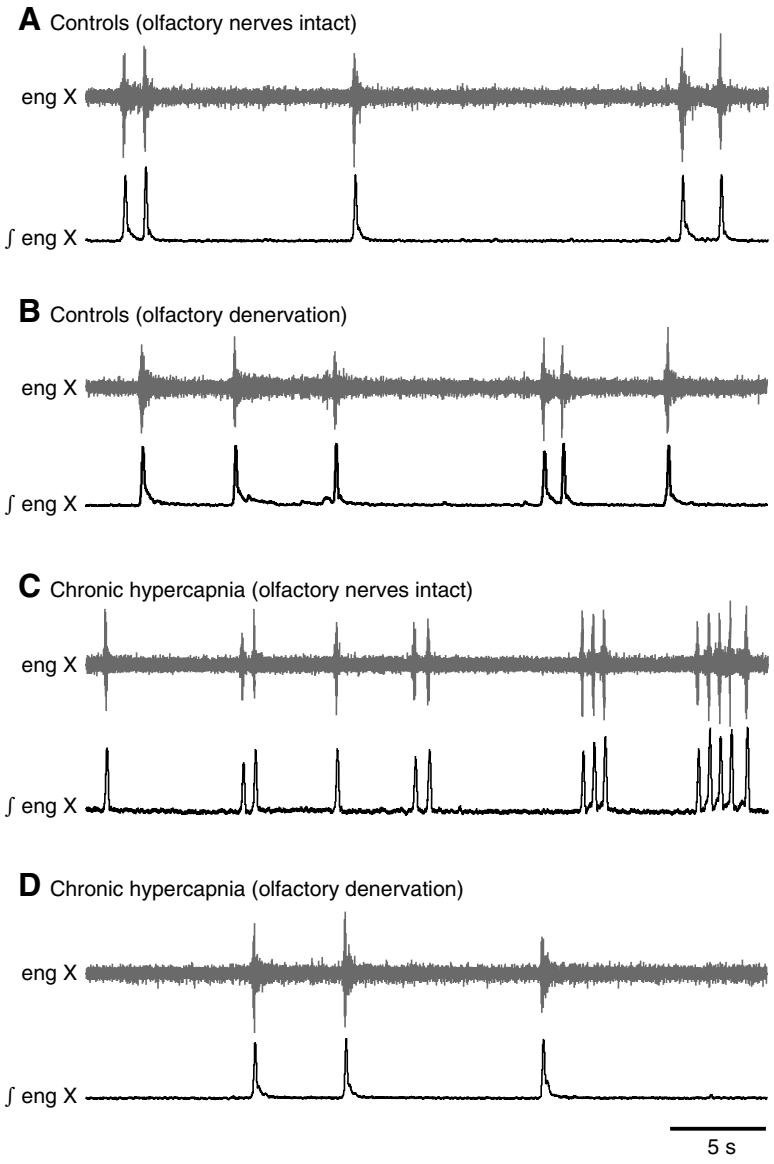

Fig. 1. Fictive breathing (vagal motor output) recorded from isolated brainstem–spinal cord preparations (at pH 7.5) taken from (A) control toads with the olfactory nerves intact, (B) control toads following olfactory denervation, (C) chronically hypercapnic toads with the olfactory nerves intact and (D) chronically hypercapnic toads following olfactory denervation. In all cases the upper trace represents the raw electroneurogram (eng X) and the lower trace (∫ eng X) represents the integrated electroneurogram.

effect on any variable in the control group (see Figs 2, 3, panels A,C,E; fictive breathing frequency, P=0.649; fictive episodes min^{-1}, P=0.080; fictive breaths per episode, P=0.154; integrated area of the fictive breath, P=0.122; total fictive ventilation index, P=0.797).

Following CHC, OD caused a reduction in fictive breathing frequency at aCSF pH levels of 7.8 and 7.5 (compare CHC OI with CHC OD in Fig. 2B; P<0.001) and a reduction in both the number of fictive episodes min^{-1} (Fig. 2D; P<0.001) and fictive breaths per episode (Fig. 2F; P<0.001) at all three aCSF pH levels. OD in the CHC group caused a reduction in the integrated area of the fictive breaths at all aCSF pH levels (Fig. 3B; P=0.002) and a decrease in the total fictive ventilation index (Fig. 3D; P<0.001) at aCSF pH levels of 7.5 and 7.8. There was no effect of OD on fictive breath duration in the CHC group (Fig. 3F; P=0.695).

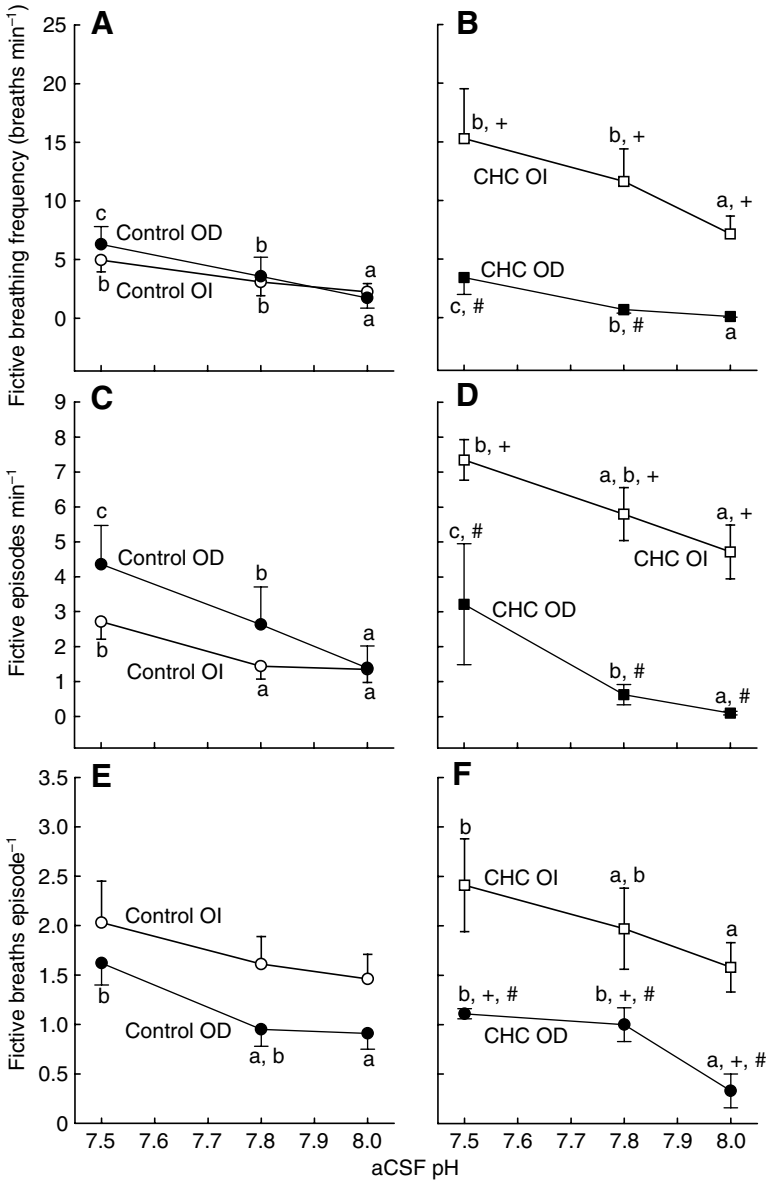

Fig. 2. The effects of chronic hypercapnia (CHC) and olfactory denervation (OD) on (A,B) fictive breathing frequency (fictive breaths min^{-1}), (C,D) the number of fictive episodes min^{-1} and (E,F) the number of fictive breaths per episode recorded from *in vitro* brainstem–spinal cord preparations taken from control (A,C,E) and CHC (B,D,F) toads. Open symbols represent preparations taken from animals with the olfactory nerves intact (OI). Filled symbols represent preparations taken from animals in which the olfactory nerves were cut (OD) prior to CHC. The data are plotted as the mean ± 1 s.e.m. Letters (a-c) represent significant differences between ventilatory parameters measured at different artificial cerebral spinal fluid (aCSF) pH levels within any group. A number sign (#) represents a difference between the OI and OD groups. A plus sign (+) represents a difference between the control and CHC groups (comparing panel A with B, C with D and E with F).

The effects of altered aCSF pH following CHO and CHH (in vitro)

The CHC data in Fig. 4 are the same data as those plotted in panels B, D and F from Figs 2 and 3 (CHC OI). They are re-plotted to facilitate comparison with the CHO and CHH data. A reduction in aCSF pH led to small but significant increases in fictive breathing frequency in both the CHO (P=0.010) and CHH (P=0.002) groups (Fig. 4A). The number of fictive episodes min^{-1} also increased, as aCSF pH was lowered, in both groups (Fig. 4B; CHO, P=0.006; CHH, P=0.002), and the number of fictive breaths per episode increased in the CHO group (P=0.010) but not in the CHH (P=0.886) group (Fig. 4C). A reduction in aCSF pH led to a decrease in the integrated area of the fictive breaths (Fig. 4D) in the CHH group (P=0.032) but not in the CHO group (P=0.438). There was a small but significant increase in the total fictive ventilation index (Fig. 4E), as aCSF pH was lowered, in the CHH group (P=0.005) but not the CHO group (P=0.055). Changes in aCSF pH had no effect on fictive breath duration in either the CHO (P=0.061) or the CHH (P=0.741) group (Fig. 4F).

The effects of CHO and CHH (in vitro)

The important consideration in this section is whether simultaneous exposure to hypercapnia and hyperoxia (i.e. CHH) reduced the increases in fictive breathing observed in the CHC group.

At all aCSF pH levels, the values for fictive breathing frequency (Fig. 4A) and the number of fictive episodes min^{-1} (Fig. 4B) were lower in the CHO and CHH groups compared with the values in the CHC group (P<0.001 in all cases). The number of fictive breaths per episode was not different in any of the groups (Fig. 4C; P=0.075). At aCSF pH levels of 7.5 and 7.8, the integrated area of the fictive breaths was reduced in the CHO group compared with the values in the CHC and CHH groups (Fig. 4D;

P<0.001). The total fictive ventilation index was reduced in the CHO and CHH groups compared with the values in the CHC group (Fig. 4E; P<0.001). There was no effect, compared with the CHC group, of either CHO or CHH on fictive breath duration (Fig. 4F; P=0.522).

In vivo experiments

The effects of altering inspired CO_2 (acute breathing trials) in vivo

In the control group (i.e. not CHC) with the olfactory nerves intact (OI) and the control group following OD, an increase in inspired CO_2 led to increases in breathing frequency (Fig. 5A; OI, P<0.001; OD, P=0.027) and the total ventilation index (Fig. 5E; OI, P<0.001; OD, P=0.006). There was an increase in the integrated area of the fictive breaths in the control OD group (Fig. 5C; P=0.017) but not in the control OI group (Fig. 5C; P=0.173).

In the CHC OI and CHC OD animals, an increase in inspired CO_2 led to increases in breathing frequency (Fig. 5B; OI, P=0.008; OD, P<0.001), the integrated area of the breaths (Fig. 5D; OI, P=0.010; OD, P=0.029) and the total ventilation index (Fig. 5F; OI, P=0.048; OD, P=0.002).

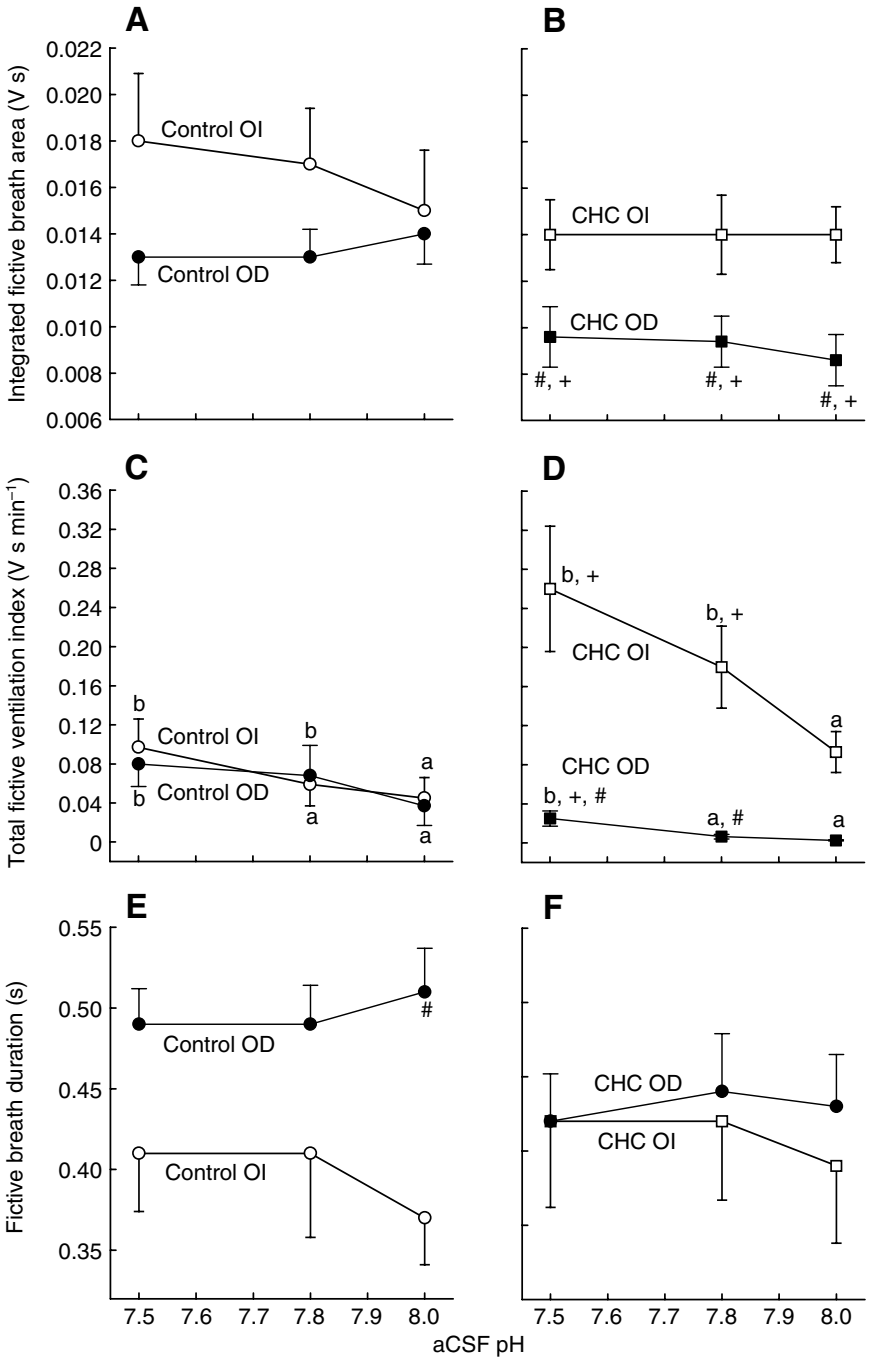

Fig. 3. The effects of chronic hypercapnia (CHC) and olfactory denervation (OD) on (A,B) integrated area of the fictive breaths (V×s), (C,D) the total fictive ventilation index (V×s min^{-1}) and (E,F) fictive breath duration (s) recorded from *in vitro* brainstem–spinal cord preparations taken from control (A,C,E) and CHC (B,D,F) toads. The data are plotted as the mean ± 1 s.e.m. Symbols and abbreviations are the same as those in Fig. 2.

The effects of CHC in vivo

With the exception of an increase in the integrated area of the fictive breaths at a CO_2 level of 2% with the olfactory nerves intact (compare Fig. 5C with Fig. 5D; open symbols; P=0.019) and an increase in the total ventilation index at a CO_2 level of 4.5% following OD (compare Fig. 5E with Fig. 5F; filled symbols; P=0.047), CHC had no effect on breathing *in vivo*.

The effects of OD in vivo

In both the control (Fig. 5A; P<0.001) and the CHC (Fig. 5B; P=0.009) groups, OD led to a reduction in breathing frequency at CO_2 levels of 2–4.5% and 2–3%, respectively. OD caused an increase in the integrated area of the breaths at 2% CO_2 in the control group (Fig. 5C; P=0.002) and at 3–4.5% CO_2 in the CHC group (Fig. 5D; P=0.006). OD led to an increase in the total ventilation index at 4.5% CO_2 in both the control (Fig. 5E; P=0.027) and CHC (Fig. 5F; P=0.020) groups.

The effects of CHH in vivo

The CHC data plotted in Fig. 6 are the same as the CHC OI data plotted in Fig. 5. Exposure to CHH abolished the increases in breathing frequency at 3 and 4.5% CO_2 (Fig. 6A; P<0.001), integrated area of the breaths at 2% CO_2 (Fig. 6B; P=0.007) and total ventilation at 2 and 3% CO_2 (Fig. 6C; P=0.004) that were observed following CHC alone.

Discussion

Fictive breathing in the *in vitro* preparations taken from toads exposed to CHC was greater than that recorded from preparations taken from control animals maintained in room air. Given this, the major finding of the current study was that the removal of respiratory-related afferent input, by OD and exposure to CHH, abolished the CHC-induced increase in central pH/CO_2-sensitive fictive breathing. The implication of these data is that respiratory-related afferent input plays a role in regulating the increase in respiratory-related central pH/CO_2 chemosensitivity that occurs during CHC.

The CHC-induced augmentation of *in vitro* fictive breathing was not manifest in the intact animals *in vivo*. During the period of CHC, the toads used for the *in vitro* and *in vivo* experiments were exposed to the same level of CO_2 (3.5%). However, during the *in vivo* experiments that followed CHC, the kidney and respiratory system are available to compensate for the respiratory acidosis that was presumably induced by the acute hypercapnic breathing trials. As such, it is possible that the level of acidosis experienced by the central chemoreceptors during the *in vivo* acute hypercapnic breathing trials was less than that experienced by the *in vitro* preparations during superfusion with hypercapnic aCSF. If this were the case, then differential buffering and the level of central acidosis could explain why the CHC-induced augmentation of breathing *in vitro* was not manifest *in vivo*. Furthermore, during the *in vivo* experiments there was also CO_2-sensitive afferent feedback from arterial chemoreceptors (stimulated by CO_2) and lung stretch receptors (inhibited by CO_2). It is possible that the presence of afferent feedback *in vivo* (and its absence *in vitro*) contributed to the different effects of CHC *in vivo* and *in vitro*.

Olfactory chemoreceptor input and central pH/CO_2 chemosensitivity

Olfactory chemoreceptors in the nasal epithelium have been shown to inhibit breathing (in acute breathing trials) when stimulated by elevated levels of CO_2 (Sakakibara, 1978; Kinkead and Milsom, 1996; Coates, 2001). This inhibition occurs at physiologically relevant CO_2 levels, and thus CO_2 is not considered to be acting as a noxious substance that would stimulate branches of the trigeminal nerve in the nasal mucosa (Coates, 2001). In the current study, we hypothesised that during exposure to CHC the olfactory chemoreceptors would be continually stimulated and therefore provide a constant source of respiratory inhibition at a time when respiratory drive was presumably elevated because of activation of central and arterial pH/CO_2-sensitive

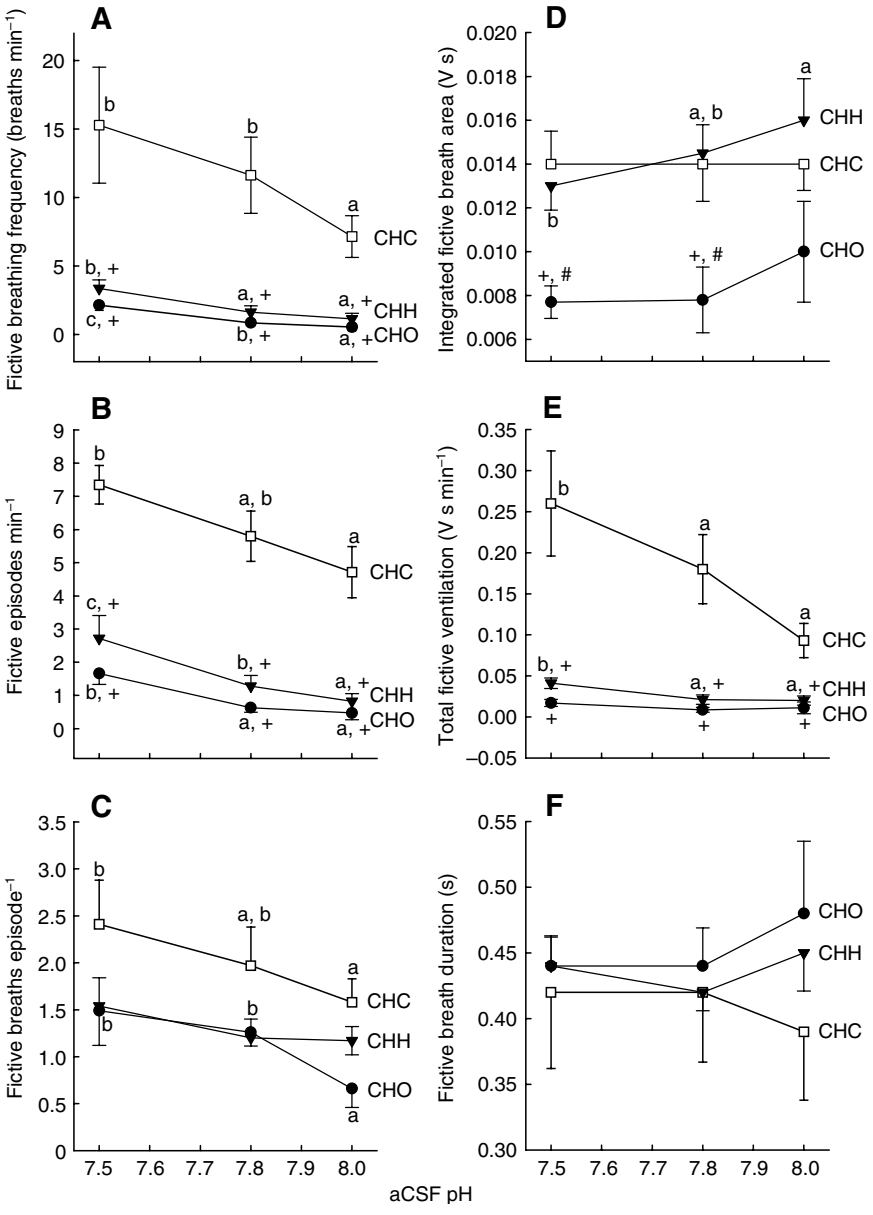

Fig. 4. The effects of chronic hypercapnia (CHC; open squares), chronic hyperoxia (CHO; filled circles) and chronic hyperoxic hypercapnia (CHH; filled triangles) on (A) fictive breathing frequency (fictive breaths min^{-1}), (B) fictive episodes min^{-1}, (C) fictive breaths per episode, (D) integrated fictive breath area (V×s), (E) total fictive ventilation index (V×s min^{-1}) and (F) fictive breath duration (s) recorded from *in vitro* brainstem–spinal cord preparations. The data are plotted as the mean ± 1 s.e.m. Letters (a-c) represent significant differences at the different artificial cerebral spinal fluid (aCSF) pH levels within any group. A plus sign (+) represents a difference from the CHC value. A number sign (#) represents a difference from the CHH value.

chemoreceptors (Smatresk and Smits, 1991; West et al., 1987). Indeed, olfactory chemoreceptors are stimulated by tonic, but not phasic, increases in upper airway CO_2 (Ballam and Coates, 1989).

The abolition of the CHC-induced augmentation of central pH/CO_2-sensitive fictive breathing by OD supports the hypothesis that the increase in central chemosensitivity occurred in order to offset continual olfactory chemoreceptor-induced inhibition of breathing that probably would have occurred during CHC. An increase in central chemosensitivity would, in turn, assist in maintaining breathing at a level appropriate for a chronic inspired CO_2 level of 3.5%. However, the possibility could have existed that olfactory chemoreceptor input to the brain would not remain at an elevated level during the period of CHC because of olfactory chemoreceptor acclimation. We are not aware of any studies that have addressed this issue. Regardless, the data do support the hypothesis and therefore the possibility of receptor acclimation does not hinder the current interpretation of the data.

In vivo, OD led to a reduction in breathing frequency in both the control and CHC groups (albeit not at all CO_2 levels). As such, OD had the same general effect on breathing frequency *in vitro* and *in vivo* in the CHC toads. However, in control toads OD had an attenuating effect on breathing frequency *in vivo* but no effect *in vitro*. One possible explanation for this is that the *in vitro* fictive breathing frequency was already relatively low and therefore could not be reduced further. *In vitro* fictive breathing recorded from isolated brainstem–spinal cord preparations in amphibians does tend to be reduced compared with breathing recorded in intact animals (Reid, 2006). This is presumably the result of a lack of afferent feedback *in vitro* (Kinkead et al., 1994; Reid et al., 2000b; Reid, 2006). In brainstem–spinal cord preparations taken from animals exposed to CHC, OD caused a reduction in the integrated area of the fictive breaths at all levels of aCSF pH. In the intact animals *in vivo*, OD caused an increase in the integrated area of the breaths at the two highest CO_2 levels. It is likely that these differences were the result of differential afferent input and or buffering capacity in the *in vitro* preparations *versus in vivo* animals, although the current data do not allow us to identify the exact basis of these differences.

Arterial chemoreceptor input and central pH/CO_2 chemosensitivity

Exposure to CHH abolished the CHC-induced increase in fictive breathing. The data therefore support the hypothesis that an increased level of arterial CO_2-sensitive chemoreceptor input (during CHC alone) may be responsible for triggering the CHC-induced increase in central chemosensitivity. Several studies support the contention that hyperoxic conditions will reduce arterial chemoreceptor feedback to the brain. First, Van Vliet and West demonstrated that, in cane toads, increased O_2 levels reduced carotid labyrinth

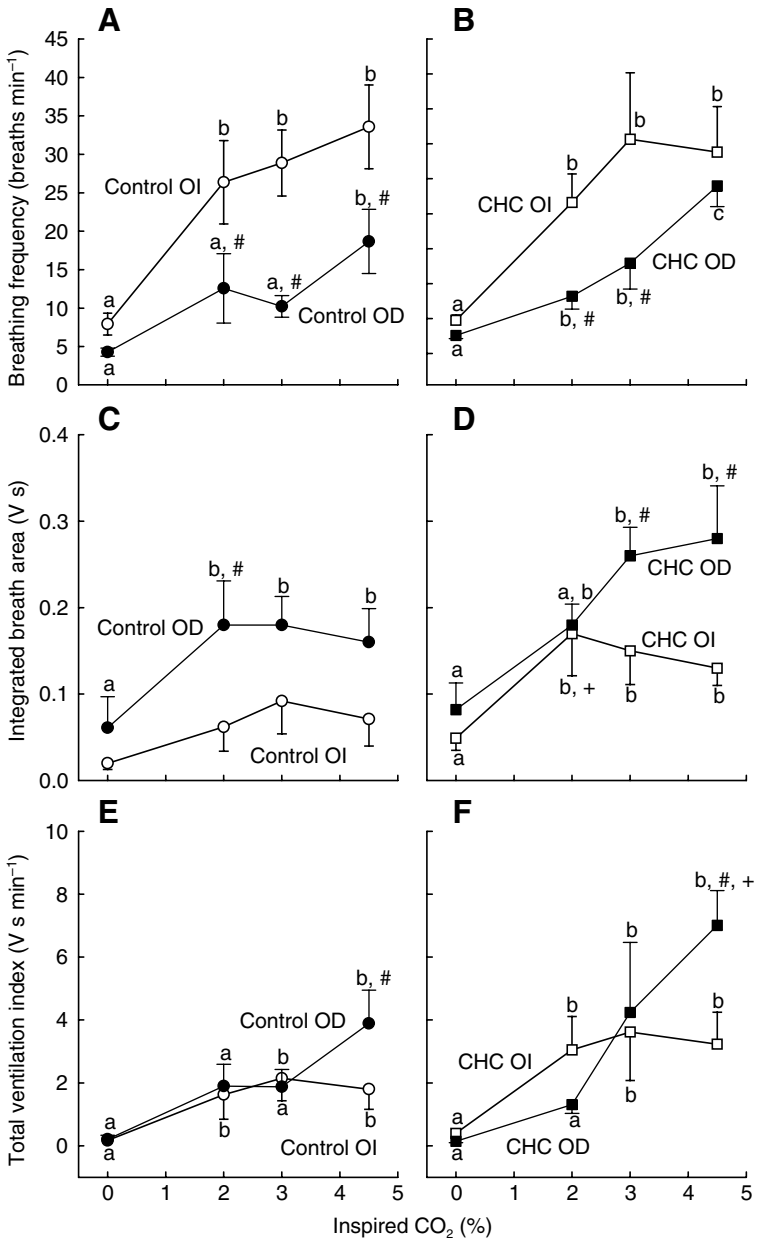

Fig. 5. (A,B) Breathing frequency (breaths min^{-1}), (C,D) integrated breath area (V×s) and (E,F) total ventilation index (V×s min^{-1}) as a function of the inspired CO_2 level recorded *in vivo* from control (A,C,E) and CHC (B,D,F) toads. Open circles (A,C,E) and open squares (B,D,F) represent animals with the olfactory nerves intact (OI). Filled circles (A,C,E) and filled squares (B,D,F) represent animals that underwent olfactory denervation (OD) prior to the 10-day period of CHC (or control conditions). The data are plotted as the mean ± 1 s.e.m. Letters (a-c) represent significant differences at the different inspired CO_2 levels within any group. A number sign (#) represents a difference between the OI and OD groups. A plus sign (+) represents a difference between the control and CHC groups (comparing panel A with B, C with D and E with F).

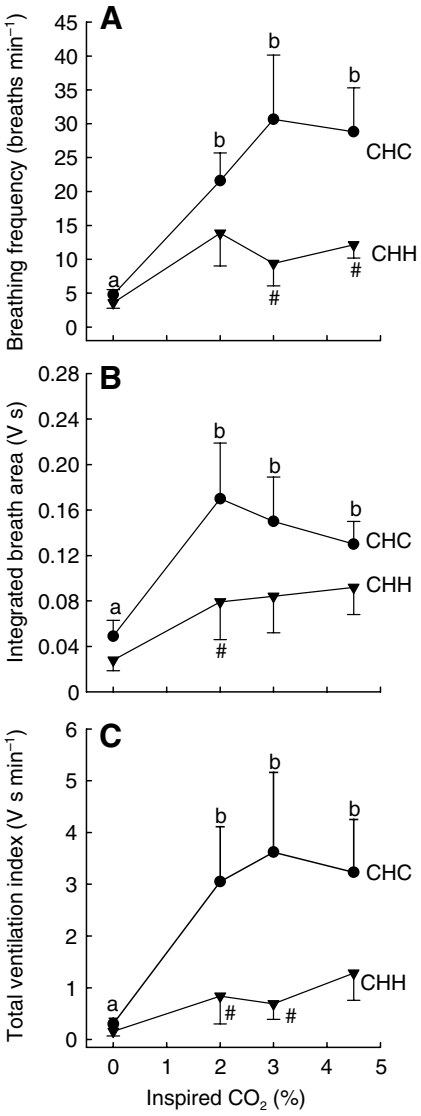

Fig. 6. (A) Breathing frequency (breaths min^{-1}), (B) integrated breath area (V×s) and (C) total ventilation index (V×s min^{-1}) as a function of inspired CO_2 levels recorded *in vivo* from toads exposed to CHC (circles) or CHH (triangles). The data are plotted as the mean ± 1 s.e.m. Letters (a-c) represent significant differences at the different inspired CO_2 levels within any group. A number sign (#) represents a difference between the CHC and CHH groups.

chemoreceptor discharge at any given level of CO_2 (Van Vliet and West, 1992). Second, Ursino et al. suggest that in humans during hyperoxia the ventilatory responses to hypercapnia are almost completely determined by the central pH/CO_2 chemoreceptors, indicating an almost complete abolition of arterial chemoreceptor feedback during hyperoxia (Ursino et al., 2001).

We also performed the *in vitro* pH/CO_2-senistivity experiments on a group of CHO (normocapnic) toads. With one minor exception, fictive breathing in the CHO group was the same as that in the CHH group. CHO alone (in the absence of CHC) had no effect on non-augmented fictive breathing, whereas CHO in conjunction with CHC (i.e. CHH) did abolish CHC-induced augmented fictive breathing. The implication of these data is that the effect of CHH is probably because of a reduction in peripheral CO_2-sensitive afferent input [which is dependent upon the O_2 level; Van Vliet and West (Van Vliet and West, 1992)] rather than a reduction in specific arterial O_2-sensitive afferent input *per se*. However, it is possible that CHO alone exerted no attenuating effect (in the absence of a CHC-induced augmentation of fictive breathing) because the levels of fictive breathing were already at a minimum and could not be reduced any further.

In the CHH experiments we made the assumption that exposure to chronic hyperoxia was reducing or abolishing afferent input from arterial chemoreceptors. However, it is possible that chronic hyperoxia had a direct effect on the central pH/CO_2 chemoreceptor cells or on central sites of pH/CO_2 chemoreceptor integration. If this were the case, then the attenuation of the CHC-induced increase in central pH/CO_2 chemosensitivity, seen following CHH, may have resulted from central effects of hyperoxia rather than a hyperoxia-induced reduction in arterial chemoreceptor input. However, all of the brainstem–spinal cord preparations were maintained under hyperoxic conditions (approximately 95–99% O_2) *in vitro*. If exposure to hyperoxia (albeit during the *in vitro* experiments rather than during a 10-day period of chronic exposure) did blunt the response of the central chemoreceptors, then it is unlikely that the CHC-induced increase in pH/CO_2-sensitive fictive breathing would have been detected in the first place.

Like the *in vitro* experiments, CHH also reduced breathing in the intact animals *in vivo*. Although the absence of a CHC-induced augmentation in breathing *in vivo* makes a comparison between the *in vivo* and *in vitro* results difficult, these *in vivo* results are consistent with the notion that reduced arterial chemoreceptor input can attenuate the hypercapnic ventilatory response.

Conclusions and perspectives

The results of this study suggest that altered afferent feedback from olfactory and arterial chemoreceptors during CHC is involved in the CHC-induced augmentation of central pH/CO_2 respiratory-related chemoreceptor function. Both OD alone and CHH alone were sufficient to abolish the augmenting effects of CHC, suggesting that altered feedback from just one of these sources is insufficient to sustain the augmented central chemosensitivity. In other words, in OD animals, enhanced arterial chemoreceptor input is not sufficient to elicit changes in central chemoreception, and enhanced feedback from olfactory chemoreceptors during CHH was not sufficient to induce the central changes in pH/CO_2-sensitive fictive breathing.

Although cane toads are generally found in tropical or neotropical environments (Pinder et al., 1992), they nevertheless can encounter chronically hypercapnic conditions in their environment. Boutilier et al. report elevated arterial

pH/CO_2 levels (approximately 2 Pa) in burrowing cane toads whose nares were open to the air while the skin was surrounded by sand at 25°C for six days (Boutilier et al., 1979b). Boutilier et al. state that it is not uncommon for cane toads to encounter hypercapnic conditions in their environment (Boutilier et al., 1979a), including hypercapnic waters in the tropics (Toews and Macintyre, 1978). Investigations such as the current study offer insight into fundamental mechanisms of respiratory plasticity and may be of value in predicting how environmental changes such as increased atmospheric CO_2 may affect physiological function.

This study was supported by NSERC of Canada Discovery and Equipment grants to S.G.R. Funding for equipment was also provided by the Canadian Foundation for Innovation, the Ontario Innovation Trust and the Ontario Thoracic Society. During the course of this study, S.G.R. held a Parker B. Francis Fellowship in Pulmonary Research (Francis Family Foundation). A.G. was the recipient of an Ontario Graduate Scholarship from the Government of Ontario.

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