Chemical activation of caudal medullary expiratory neurones alters the pattern of breathing in the cat

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1. The purpose of this work was to ascertain whether the activation of caudal expiratory neurones located in the caudal part of the ventral respiratory group (VRG) may affect the pattern of breathing via medullary axon collaterals.

2. We used microinjections of DL-homocysteic acid (DLH) to activate this population of neurones in pentobarbitone-anaesthetized, vagotomized, paralysed and artificially ventilated cats. Both phrenic and abdominal nerve activities were monitored; extracellular recordings from medullary and upper cervical cord respiratory neurones were performed.

3. DLH (160 mM) microinjected (10–30 nl for a total of 1·6–4·8 nmol) into the caudal VRG, into sites where expiratory activity was encountered, provoked an intense and sustained activation of the expiratory motor output associated with a corresponding period of silence in phrenic nerve activity. During the progressive decline of the activation of abdominal motoneurones, rhythmic inspiratory activity resumed, displaying a decrease in frequency and a marked reduction or the complete suppression of postinspiratory activity as its most consistent features.

4. Medullary and upper cervical cord inspiratory neurones exhibited inhibitory responses consistent with those observed in phrenic nerve activity, while expiratory neurones in the caudal VRG on the side contralateral to the injection showed excitation patterns similar to those of abdominal motoneurones. On the other hand, in correspondence to expiratory motor output activation, expiratory neurones of the Bötzinger complex displayed tonic discharges whose intensity was markedly lower than the peak level of control breaths.

5. Bilateral lignocaine blockades of neural transmission at C2–C3 affecting the expiratory and, to a varying extent, the inspiratory bulbospinal pathways as well as spinal cord transections at C2–C3 or C1–C2, did not suppress the inhibitory effect on inspiratory neurones of either the ipsi- or contralateral VRG in response to DLH microinjections into the caudal VRG.

6. The results show that neurones within the column of caudal VRG expiratory neurones promote inhibitory effects on phrenic nerve activity and resetting of the respiratory rhythm. We suggest that these effects are mediated by medullary bulbospinal expiratory neurones, which may, therefore, have a function in the control of breathing through medullary axon collaterals.

Synaptic interactions between respiratory neurones located in the lower brainstem, particularly in the medulla oblongata, probably underlie the genesis of respiratory rhythm in mammals (for reviews see e.g. Long & Duffin, 1986; von Euler, 1986).

Most of the expiratory neurones located in the caudal part of the ventral respiratory group (VRG) are bulbospinal neurones (see e.g. Merrill, 1974) phasically inhibited during inspiration (Mitchell & Herbert, 1974; Bainton & Kirkwood, 1979; Ballantyne & Richter, 1986). They receive their excitatory input from more rostral regions of the medulla (Merrill, 1979; Speck & Beck, 1989). The origin of the various synaptic inputs responsible for the rhythmic activity of caudal expiratory neurones has not been definitely determined. The ramp-like development of their expiratory discharge might result from a rhythmic excitatory drive arising from a more rostral population of expiratory neurones (the so-called Bötzinger complex) located in the

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vicinity of the retrofacial nucleus. This has been suggested by antidromic mapping studies (Fedorko & Merrill, 1984) and by recent findings obtained by means of electrical and chemical activation of these rostral expiratory neurones (Bongianni, Corda, Fontana & Pantaleo, 1990). However, it could also result from a tonic excitatory drive (Bainton & Kirkwood, 1979) patterned by rhythmic inhibitory inputs originating in different types of inspiratory neurones as well as in postinspiratory neurones located in the rostral VRG or in the region of the retrofacial nucleus (Anders, Ballantyne, Bischoff, Lalley & Richter, 1991).

Both electrophysiological (Merrill, 1974, 1979; Lipski, Trzebski, Chodobska & Kruk, 1984) and morphological (Kreuter, Richter, Camerer & Senekowitsch, 1977; Kalia, 1981; Arita, Kogo & Koshiya, 1987) lines of evidence indicate that caudal expiratory neurones are probably not involved in the respiratory rhythmogenesis since they do not have medullary axon collaterals and, therefore, lack appropriate connections with other medullary respiratory neurones (for reviews see Long & Duffin, 1986; von Euler, 1986). Furthermore, transection or lesion studies (Merrill, 1979; Huang & St John, 1988; Speck & Beck, 1989) have led to similar conclusions. However, before definitely rejecting any role of caudal expiratory neurones in respiratory rhythm generation or, at least, in partially shaping the pattern of breathing, other functional evidence should be provided, such as that arising from elevating the level of activity of these neurones rather than eliminating it as happens in lesion or transection experiments.

The present study was designed to determine if the pattern of breathing as well as the activity of medullary and upper cervical cord respiratory neurones (Long & Duffin, 1986; von Euler, 1986) are affected by the activation of this population of neurones. This investigation was carried out by means of microinjections of the broad-spectrum excitatory amino acid agonist DL-homocysteic acid (DLH), which excites cell bodies, but not axons of passage (Zieglgansberger & Puil, 1973; Goodchild, Dampney & Bandler, 1982). To ensure that DLH-induced effects were related to medullary rather than to spinal cord neural mechanisms, an attempt was also made to test the response of VRG inspiratory neurones to DLH microinjections into the caudal expiratory part of the VRG both before and after lignocaine blockades of bulbospinal respiratory pathways or spinal cord transections.

A preliminary account of part of the present results has previously been published in abstract form (Pantaleo, Bongianni, Fontana & Corda, 1990).

METHODS

General animal preparation

Experiments were performed on thirty-two adult cats of both sexes weighing 2·3–4·1 kg, initially anaesthetized with sodium pentobarbitone (35 mg kg^{-1} I.P.; subsequently maintained with supplementary intravenous doses of 2–4 mg kg^{-1} h^{-1}). Atropine (0·1 mg kg^{-1} I.M.) and dexamethasone (2 mg kg^{-1} I.V.) were administered to reduce mucosal secretion in the airways and brain oedema, respectively.

The trachea was cannulated. Catheters were inserted into a femoral artery and vein for monitoring arterial pressure and for drug delivery, respectively. Both C5 cervical branches of the phrenic nerve and medial branches of the first (L1) or the second (L2) lumbar nerves to the abdominal muscles were dissected free, cut distally and prepared for recordings. Both vagus nerves were cut at mid-cervical level. The internal branches of the superior laryngeal nerves (SLN) were also exposed bilaterally and prepared for electrical stimulation.

The animal was fixed in a stereotaxic apparatus with the head ventroflexed and was supported by dorsal clamps in the thoracic and lumbar areas of the spine. The dorsal surface of the medulla was widely exposed by occipital craniotomy and the dura and arachnoid membranes were removed. The posterior part of the cerebellum was either retracted or removed by aspiration to provide access to the rostral part of the medulla. The upper cervical spinal cord segments were also exposed by laminectomy.

The animals were paralysed with gallamine triethiodide (5 mg kg^{-1} I.V., supplemented at 2 mg kg^{-1} h^{-1}) and artificially ventilated with oxygen-enriched room air. Ventilatory volume and rate were adjusted to maintain end-tidal CO_2 at 4–5 %. Rectal temperature was kept at $37 \pm 0{\cdot}5$ °C by a servo-controlled heating pad.

Throughout the course of these experiments, the depth of anaesthesia was assessed by monitoring a stable and regular pattern of phrenic discharge as well as by the absence of fluctations in arterial blood pressure (mean pressure less than 150 mmHg) whether spontaneous or in response to nociceptive stimuli. The supplementary doses of sodium pentobarbitone were adjusted to maintain a stable level of anaesthesia.

Recording procedures

The C5 phrenic roots and abdominal nerves (L1 or L2) were de-sheathed and mounted on bipolar platinum electrodes immersed in mineral oil or covered in semi-solid paraffin. Efferent phrenic activities were differentially amplified (×2000–10 000), bandpass filtered (80–10 000 Hz), full-wave rectified and integrated (lowpass RC filter, time constant adjustable from 20 to 200 ms, usually set at 100 ms). Extracellular recordings from medullary or upper cervical cord respiratory neurones were made with tungsten microelectrodes (5–12 MΩ impedance, as tested at 1 kHz). Recorded discharges from single neurones or small groups of neurones were amplified (×1000–5000), bandpass filtered (50–5000 Hz), processed and integrated in the same way as phrenic and abdominal nerve activities. To discriminate extracellular spikes before further processing, a window discriminator, which provided an output of standard pulses, was used in some instances. Extracellular neuronal activity was recorded from the rostral inspiratory portion of the VRG (0·5–3 mm rostral to the obex, 3·0–3·6 mm lateral to the midline, and 3·5–5 mm below the dorsal surface of the medulla), from the Bötzinger complex (4·0–5·5 mm rostral to the obex, 2·9–3·5 mm lateral to the mid-line, and 4·0–5·5 mm below the dorsal medullary surface) and from the most caudal expiratory part of the VRG (1·5–4·2 mm caudal to the obex, 2·5–3·2 mm lateral to the mid-line, and 2·5–3·5 mm below the dorsal medullary surface). In a few preparations ($n = 3$) recordings were also obtained from inspiratory neurones of the upper cervical spinal cord at C1–C2 (see e.g. Aoki, Mori, Kawahara, Watanabe & Ebata, 1980; Lipski & Duffin, 1986). The dorsal root entry zone was used as a guide in locating the inspiratory neurones; recordings were made 2·0–3·5 mm lateral to the mid-line at a depth of 1·8–2·5 mm below the dorsal surface.

Extracellular action potentials were in all probability recorded from cell bodies since they displayed biphasic or triphasic shapes, relatively high amplitudes (100–300 μV) and long durations ($\geqslant$ 1 ms). In addition, recordings were stable and could be held over a long (50–100 μm) microelectrode travel (see Nelson, 1959; Kirkwood, Munson, Sears & Westgaard, 1988). In some experiments ($n = 8$) the recording sites were marked with small cathodal electrolytic lesions (20 μA for 20–30 s) to facilitate later histological identification.

Efferent phrenic and abdominal nerve activities as well as neuronal activity were monitored as 'raw' signals on an oscilloscope (Model 5112, Tektronix, Beaverton, OR, USA) and fed to an audiomonitor. Strain-gauge manometers were used for monitoring arterial blood pressure and intratracheal pressure. End-tidal partial pressure of CO_2 was measured by an infrared CO_2 analyser (Datex CD-102, Normocap, Helsinki, Finland). Integrated phrenic, abdominal and neuronal activities as well as the signals of the other variables studied were recorded on an eight-channel chart recorder (Model 8K20, Nec San-ei, Tokyo, Japan).

Stimulation procedures

Rectangular pulses (0·5 ms duration) were delivered by means of a stimulus isolation unit (SIU5, Grass Instruments, Quincy, MA, USA) driven by a stimulator (Grass S8). Current intensity was monitored as voltage drop through a small series resistor by using a differential amplifier and an oscilloscope. For SLN stimulation bipolar silver electrodes were used. Short (2–4 s) trains of stimuli at 20–50 Hz at an intensity capable of activating abdominal motoneurones (as a rule in the range 50–100 μA, i.e. 5–6 times the threshold intensity for inhibition of phrenic nerve activity) were delivered during expiration (Bongianni, Corda, Fontana & Pantaleo, 1988).

Chemical activation of the caudal expiratory portion of the VRG was achieved using microinjections (10–30 nl) of 160 mM DLH. The drug was dissolved in 0·9 % NaCl solution or in artificial cerebrospinal fluid (Mitchell, Loeschcke, Massion & Severinghaus, 1963); the pH was adjusted to 7·4 with 1 N NaOH. The injections were performed via a glass micropipette (tip diameter 10–25 μm) by applying pressure using an air-filled syringe connected to the micropipette by polyethylene tubing. The micropipette was glued to a tungsten microelectrode (similar to those employed for extracellular recordings) whose tip protruded 100–150 μm from the pipette tip. In this way it was possible to apply chemical stimulation to the same area from which neuronal activity was recorded. Injections were made in the caudal VRG in sites where intense multiunit expiratory activity was recorded. Control injections of saline or artificial cerebrospinal fluid were also performed in the same area.

The time taken to inject the solution ranged from 5 to 10 s. The volume of each injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a microscope equipped with a fine reticule.

Lignocaine blockades of bulbospinal neural transmission and spinal cord transections

Bilateral microinjections (0·5–1 μl) of a 4 % (w/v) lignocaine hydrochloride solution were performed in the ventral columns of the spinal cord at C2–C3 to block neural transmission especially in bulbospinal pathways mediating the expiratory drive to expiratory motoneurones. At this level of the spinal cord bulbospinal inspiratory and expiratory pathways appear to be separated, the latter lying more medially in the ventromedial white matter, ventral to the ventral horns (Newsom Davis & Plum, 1972; Merrill, 1979; for review see Long & Duffin, 1986). We used the microinjection technique already described for chemical stimulation. The dorsal root entry zone (or the dorsolateral sulcus) was used as a reference point. Penetrations 1·0–1·5 mm medial to this zone were designed to reach the ventral spinal columns. The injecting electrode was positioned perpendicular to the spinal cord. After insertion and penetration to 2 mm depth, it was advanced in discrete increments of 0·25 mm; at each step the orthodromic response of abdominal motoneurones to single current pulses (0·5 ms, 20–50 μA) delivered during expiration was evaluated. The sites at which the maximal orthodromic abdominal responses could be evoked were judged suitable for the injection (as a rule from 3·5–4·5 mm below the dorsal surface of the spinal cord). The effectiveness of the neural block was assessed by the cessation of spontaneous expiratory activity (when it was present in control conditions) and especially by the elimination of the reflex activation of abdominal motoneurones in response to SLN stimulation (Bongianni *et al.* 1988). The neural block was subsequently confirmed by the absence of expiratory motor output activation in response to DLH injected into the caudal VRG (see Results). Due to the spread of the injectate to neighbouring areas of the ventrolateral spinal columns where inspiratory axons are located (Newsom Davis & Plum, 1972; for review see Long & Duffin, 1986), often even phrenic nerve activity was attenuated or completely suppressed.

Spinal cord transections at C1–C2 or at C2–C3 were made under microscope control. After removal of the dura mater the cord was sectioned with a narrow slip of razor blade cemented into a fine pair of forceps. This manoeuvre led, as expected, to the disappearance of phrenic nerve activity. Cutting the spinal cord, unlike lignocaine blockade, led to marked falls in arterial blood pressure, which were counteracted by the infusion of 6 % dextran and injections of metaraminol bitartrate. At the end of these experiments we examined the region of transection to ensure that it was complete. In lignocaine blockade or transection trials the main objective was to succeed in maintaining recordings from the same VRG inspiratory neurones, especially in the absence of the inspiratory motor output, both before and after lignocaine blockade or spinal cord transection, as well as to retest the response to DLH injected into the same caudal VRG location.

Histology

At the end of the experiment the brain was perfused with 0·9 % NaCl solution and then with 10 % formalin solution via a carotid artery. After at least 48 h immersion in 10 % formalin solution the brain was placed in a hypertonic sucrose solution. Frozen serial sections (50 μm) were made in the frontal plane to confirm the stereotaxically located recording and injection sites, according to the atlas of Berman (1968).

Data analysis

Measurements were performed on paper recordings (usual paper speed 2–5 mm s^{-1}, occasionally 10–25 mm s^{-1}). The respiratory frequency (breaths min^{-1}) and peak amplitude (arbitrary units) of the integrated phrenic nerve and abdominal nerve activity were measured. The slope of the straight line drawn from the onset to 90 % of the maximum level of the phrenic ramp was taken as a reliable estimate of the inspiratory rate of rise. Respiratory variables were measured for an average of five consecutive breaths in the period immediately preceding each

trial (control values). The average value for each period was treated as a single measurement for the purpose of analysis. Since DLH microinjections caused an apnoeic period in the inspiratory activity (see Results), this period was evaluated together with the respiratory variables of the first recovery breath. Statistical analysis was made by means of Student's paired t tests or the Wilcoxon signed rank test. A P value $< 0{\cdot}05$ was considered to be statistically significant. Changes in respiratory variables were expressed as percentage variations of control values. All values are presented as means ± standard error of the mean.

RESULTS

General features of DLH-induced responses

DLH (160 mM) injected into the caudal VRG (82 microinjections, 10–30 nl, 1·6–4·8 nmol) in sites where intense multiunit expiratory activity was recorded, consistently produced an immediate and sustained non-rhythmic (tonic) activation of the expiratory motor output associated with a corresponding period of inhibition in phrenic nerve activity (mean duration 16·8 ± 1·2 s; range 5–58 s) and resetting of the respiratory rhythm. Phrenic nerve activity was either completely silenced or presented a very low level of tonic activity with small superimposed oscillations. A typical example of these effects is illustrated in Fig. 1. Rhythmic respiratory activity resumed, as a rule, during the progressive decline of the activation of abdominal motoneurones and recovered progressively (Figs 1 and 2). Varied effects were observed on respiratory activity during the initial part of the recovery period, without any obvious relation to the localization of the injection site in the caudal VRG or to the volume of the injectate. One of the most consistent features ($n = 54$) was a reduction in respiratory frequency (range 2·8–62 %) as evaluated on the first recovery breath, even though in some cases we observed either no changes ($n = 12$) or increases (range 3–75 %; $n = 16$) in respiratory frequency. The overall decrease in frequency was 14·3 ± 2·6 %, from baseline values of 14·9 ± 0·2 to 12·6 ± 0·4 breaths min^{-1} ($n = 82$; $P < 0{\cdot}0001$). An apneustic-like pattern was not uncommon ($n = 22$) at the beginning of the recovery period (e.g. Figs 1 and 2). In addition, post-inspiratory activity, when evident in the control conditions, always markedly decreased or completely disappeared ($n = 42$) in the early postapnoeic breaths (Fig. 3), and then displayed progressive recovery.

Peak phrenic amplitude of the first recovery breath could be similar ($n = 21$) to that of control breaths or even increased (range 10–76·5 %; $n = 27$), but it was in most instances depressed (range 12·5–52·4 %; $n = 34$). This variable showed a mean decrease of 4·2 ± 2·8 %, which did not reach the level of statistical significance ($n = 82$; $P > 0{\cdot}05$). Similarly, no consistent changes were observed in the rate of rise of phrenic nerve activity (mean decrease 2·6 ± 1·8 %; $n = 82$; $P > 0{\cdot}05$). Sometimes ($n = 16$) the initial depression in phrenic nerve activity was followed by a period of enhanced inspiratory activity, mainly due to an increase in peak amplitude, before complete recovery (Fig. 1).

All the observed effects were bilaterally symmetrical in the inspiratory, but not in the expiratory motor output; a more pronounced activation was observed, as a rule, in the

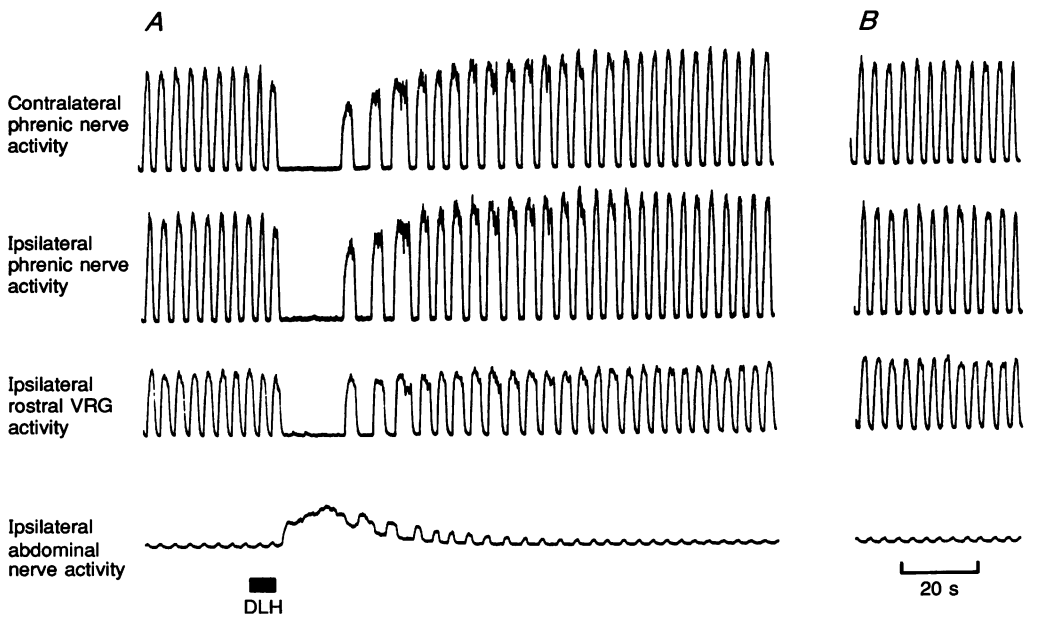

Figure 1. Example of respiratory response to 15 nl of DLH (160 mM) injected into the caudal expiratory region of the VRG

A, control and effects immediately following the injection (bar). *B*, 18 min after completion of the injection. Traces from top to bottom: contralateral and ipsilateral integrated phrenic nerve activity, integrated activity of an inspiratory neurone of the ipsilateral rostral VRG and integrated ipsilateral abdominal nerve activity.

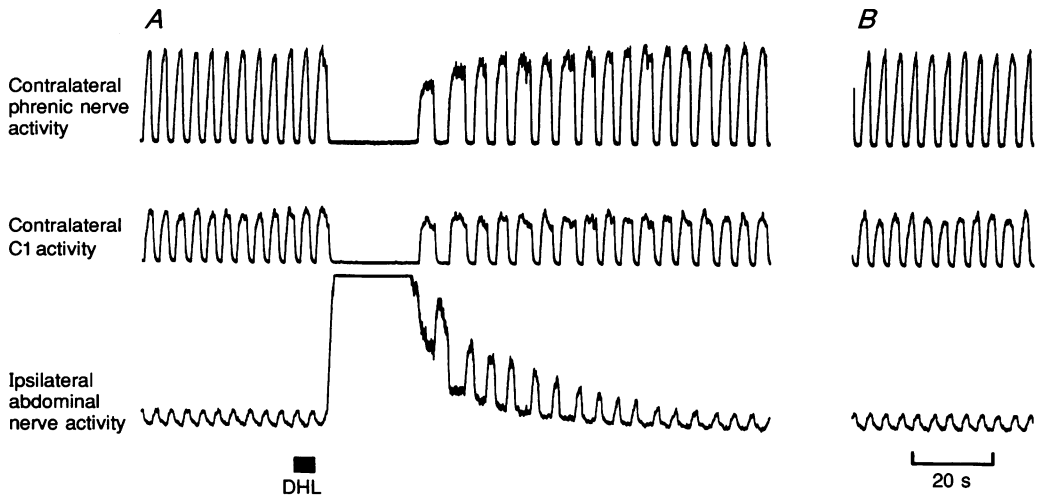

Figure 2. Example illustrating the response of upper cervical cord inspiratory neurones to 20 nl of DLH (160 mM) injected into the expiratory caudal part of the VRG
A, control and effects of the injection (bar); *B*, 12 min after the end of the injection. Traces from top to bottom: contralateral integrated phrenic nerve activity, integrated activity of a single inspiratory neurone at the contralateral C1 level and ipsilateral integrated abdominal nerve activity. Note saturation in the recording of abdominal nerve activity during the excitatory response.

abdominal motoneurones contralateral to the injection site (Figs 6 and 7). Differences in peak expiratory activity ranged from 22 to 205 % with a mean value of 67·4 ± 8·2 % ($n = 48$; $P < 0{\cdot}0001$). No obvious or consistent changes in arterial blood pressure accompanied these respiratory responses (Figs 4, 6 and 7); in a few instances slight (≤ 20 mmHg) increases ($n = 10$) or decreases ($n = 14$) were observed.

The onset of the effects was rapid (5–10 s from the beginning of the injection) and a complete recovery was observed within 15 min. The responses to DLH micro-injections were reproducible at the same site 15–20 min after the preceding injection. Control injections of equivalent volumes of 0·9 % NaCl solution or artificial cerebrospinal fluid were completely ineffective as were DLH injections in

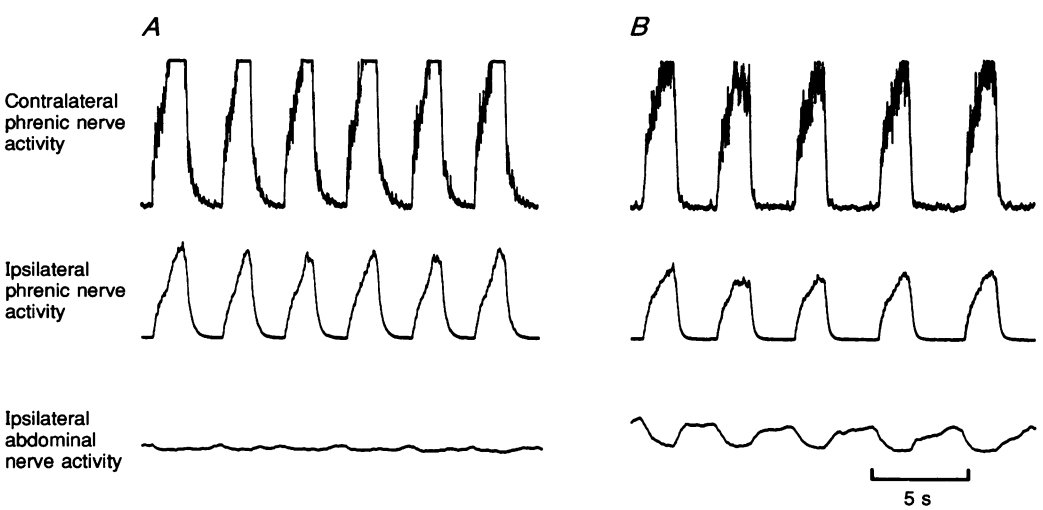

Figure 3. Marked depression of postinspiratory activity during the activation of the expiratory motor output following an injection (10 nl) of DLH (160 mM) into the caudal VRG
A, control; *B*, 30 s after completion of the injection. Traces from top to bottom: contralateral and ipsilateral integrated phrenic nerve activity, ipsilateral integrated abdominal nerve activity. The contralateral integrated phrenic activity has been recorded at higher amplification (note saturation before peak phrenic activity) and lower time constant (35 ms) to provide a more detailed display of changes in postinspiratory activity.

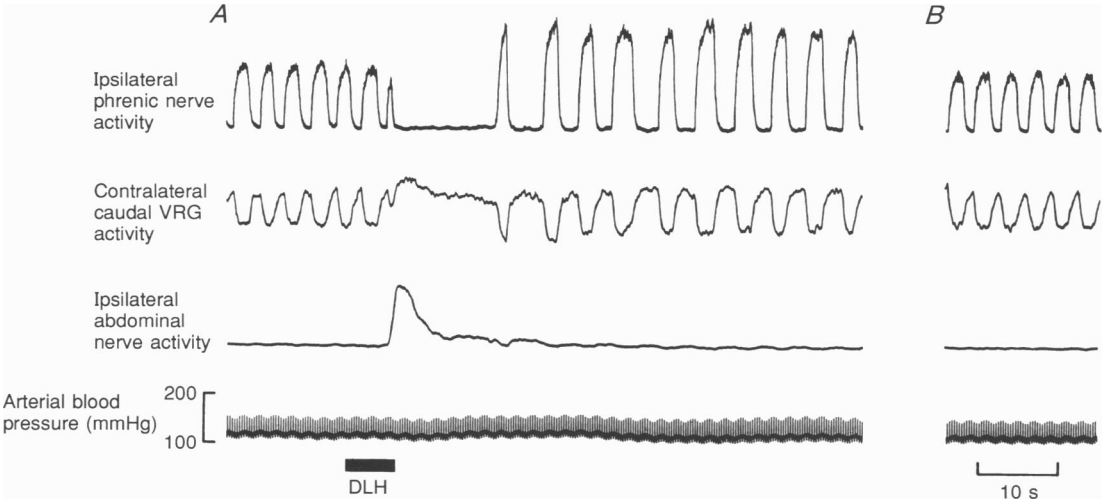

Figure 4. Sustained activation of expiratory neurones in the contralateral caudal VRG in response to 15 nl of DLH (160 mM) injected into the caudal VRG
A, control and DLH-induced respiratory effects; *B*, 10 min after completion of the injection. Traces from top to bottom: ipsilateral integrated phrenic nerve activity, integrated expiratory activity recorded from a small group of neurones in the contralateral caudal VRG, ipsilateral integrated abdominal nerve activity and arterial blood pressure. Injection is marked by a bar.

sites 0·3 mm or more away from those where expiratory activity in the caudal VRG had been encountered.

Responses in medullary and upper spinal cord respiratory neurones

Recordings from both single cells and small groups of neurones were used in the evaluation of neuronal response to DLH injections. DLH-induced effects on the activity of respiratory neurones were consistent with those observed in the inspiratory and expiratory motoneurones. The activity recorded from augmenting inspiratory neurones of the ipsilateral (5 single unit and 3 pauci-unit recordings) and contralateral VRG (7 single unit and 9 pauci-unit recordings) showed a behaviour similar to that of phrenic nerve activity

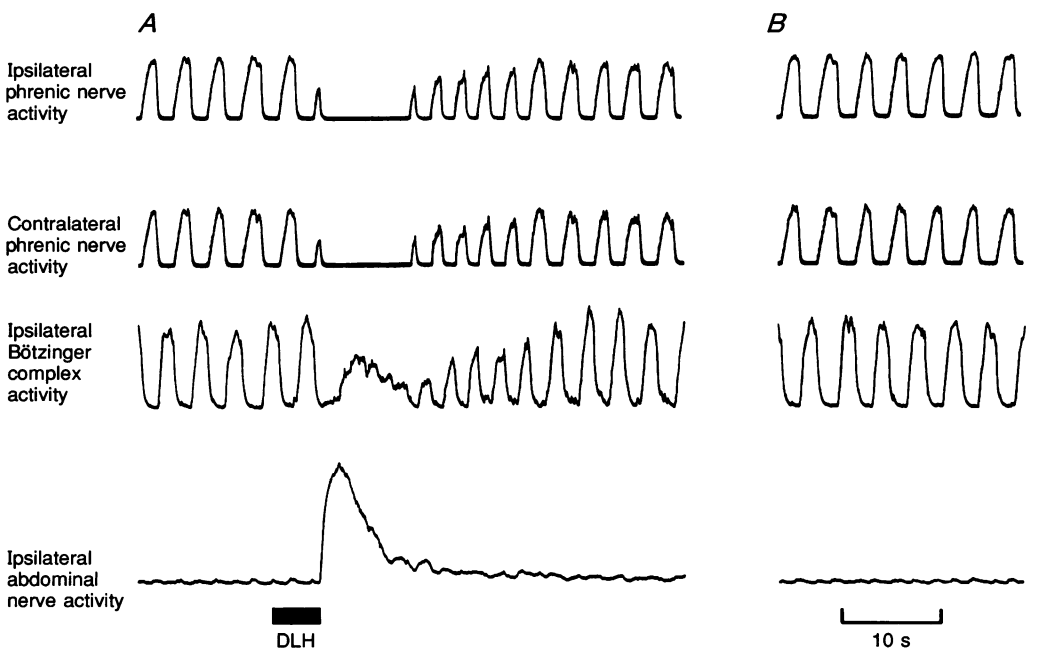

Figure 5. Example of changes in the activity of Bötzinger complex expiratory neurones in response to 10 nl of DLH (160 mM) injected into the caudal VRG
A, control and effects of the injection (bar); *B*, 15 min after the end of the injection. Traces from top to bottom: ipsilateral and contralateral integrated phrenic nerve activity, integrated pauci-unit expiratory activity recorded from the ipsilateral Bötzinger complex and ipsilateral integrated abdominal nerve activity.

(Fig. 1). Similar features were displayed by augmenting inspiratory neurones encountered in the ipsilateral (1 single unit and 2 pauci-unit recordings) and contralateral (2 single unit and 4 pauci-unit recordings) DRG. In a few recordings (3 single unit and 1 pauci-unit recordings) from upper cervical cord inspiratory neurones (C1–C2) contralateral to the side of the injection, we observed neuronal responses to DLH microinjections which were similar in all respects to those displayed by phrenic motoneurones (Fig. 2).

Expiratory neurones in the caudal VRG contralateral to the side of the injection (2 single unit and 5 pauci-unit recordings) presented excitatory phenomena which were consistent with the pattern of activation observed in expiratory motoneurones (Fig. 4). Recordings from expiratory neurones of the Bötzinger complex population, both ipsilateral (3 pauci-unit recordings) and contralateral (3 single unit and 3 pauci-unit recordings) to the side of the injection, did not display patterns of neuronal response which corresponded to those of the expiratory motor output. Indeed, all tested neurones showed tonic discharge patterns, often with superimposed phasic bursts, in correspondence to the activation of expiratory motoneurones. However, the intensity of this tonic activity was always markedly lower than the peak level of control breaths. In addition, in most instances these neurones displayed a very low discharge rate or even a complete silence in correspondence to the peak activity of the expiratory motor output. When rhythmic respiration resumed, the activity of rostral expiratory neurones was depressed (reduced peak level) and recovered progressively to control levels (Fig. 5).

Lignocaine blockade and transection of the spinal cord

Lignocaine bilateral blockade of neural transmission in the respiratory bulbospinal descending pathways at C2–C3 (6 successful experiments) suppressed spontaneous expiratory activity and the reflex activation of expiratory motoneurones in response to high-intensity SLN stimulation (Bongianni *et al.* 1988). Neural transmission in bulbospinal inspiratory axons was only partially blocked in most cases ($n = 4$) so that, even if reduced, some phrenic nerve activity was still present; a complete block was achieved in the other preparations ($n = 2$). We were able to maintain recordings from the same inspiratory neurones of the VRG ipsilateral (1 pauci-unit recording) or contralateral (1 single unit and 5 pauci-unit recordings) to the side of the injection both before and after lignocaine blockade, and to retest their response to chemical stimulation in the same location of the caudal VRG. After lignocaine blockade, DLH microinjections still provoked a pause in neuronal and phrenic nerve activity (when present) without any concomitant activation of the

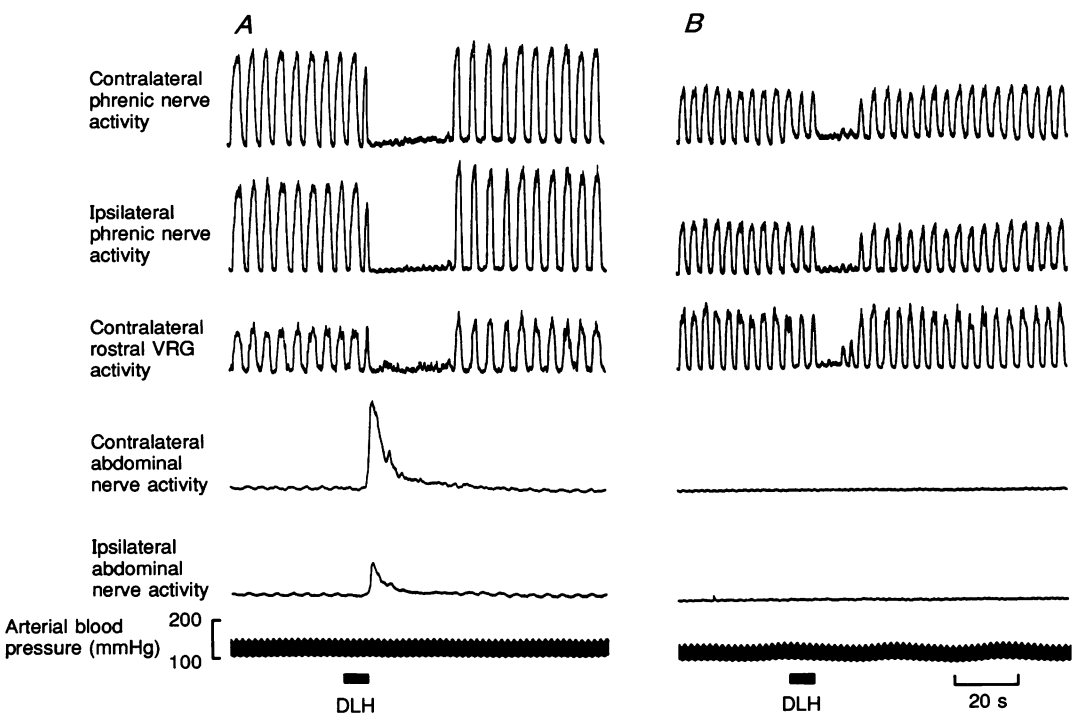

Figure 6.
Changes in the inspiratory and expiratory motor output as well as in the activity of a small population of inspiratory neurones of the contralateral rostral VRG in response to 15 nl of DLH (160 mM) injected into the same site in the caudal VRG before (*A*) and after (*B*) lignocaine bilateral blockade of neural transmission in the respiratory bulbospinal pathway at C2–C3. Also the bulbospinal inspiratory descending pathway was partially blocked, as revealed by a reduction in peak phrenic nerve activity. Traces from top to bottom: contralateral and ipsilateral integrated phrenic nerve activity, integrated pauci-unit activity of inspiratory neurones of the contralateral rostral VRG, contralateral and ipsilateral integrated abdominal nerve activity and arterial blood pressure. Injections marked by bars.

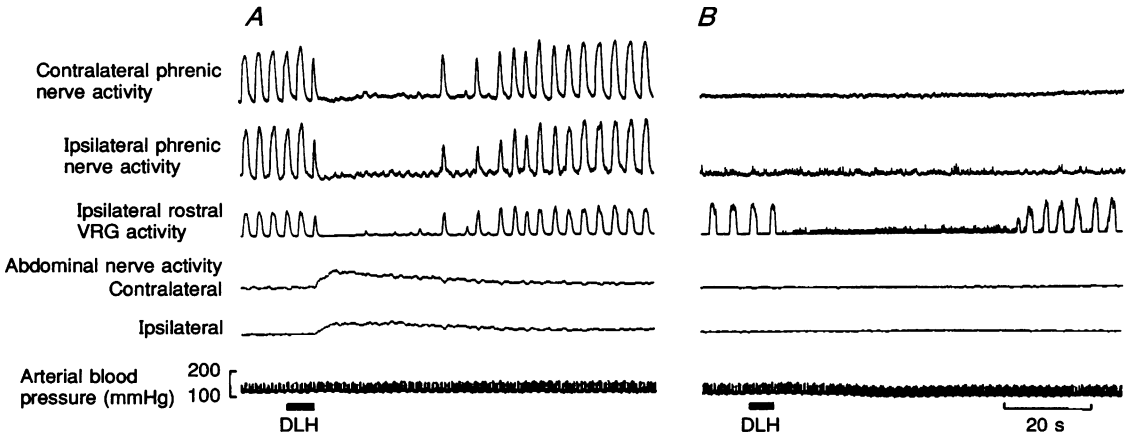

Figure 7. Effects induced by a microinjection (20 nl) of DLH (160 mM) into the same site in the caudal VRG on respiratory activity before (*A*) and after (*B*) a spinal transection between C1 and C2

Traces from top to bottom: contralateral and ipsilateral integrated phrenic nerve activity, integrated activity of a single inspiratory neurone of the ipsilateral rostral VRG, contralateral and ipsilateral integrated abdominal nerve activity and arterial blood pressure. Injections are marked by bars.

expiratory motor output (Fig. 6). Blockade of neuronal transmission turned out to be reversible within 40–80 min, at least to a large extent.

Spinal cord transection experiments were successfully performed in three cats: in one of them at C1–C2 and in the others at C2–C3. Spinal cord transections did not eliminate DLH-induced inhibitory effects on the activity of ipsilateral (2 single unit recordings) or contralateral (1 pauci-unit recording) VRG inspiratory neurones (Fig. 7).

DISCUSSION

This study is the first to provide evidence that the activation of neurones located in the caudal part of the VRG causes strong excitatory effects in the expiratory motor output and alters the pattern of breathing by inducing inhibitory effects in the activity of phrenic motoneurones as well as in that of medullary and upper cervical cord inspiratory neurones. We suggest that these effects are mediated by caudal VRG bulbospinal expiratory neurones through axon collaterals.

We are confident that the respiratory effects were confined to responsive sites localized within the most caudal part of the VRG. Only DLH injections in sites where strong multiunit expiratory activity was recorded produced the characteristic effect we have reported. In an attempt to restrict the spread of the injectate (and hence the number of neurones excited) only injections of relatively small volumes of DLH solution were considered (as a rule 10–20 nl, and only on a few occasions up to 30 nl). Theoretical calculations by Nicholson (1985) suggest that volumes of 10–20 nl should spread less than 300 μm in any direction from the injection site, and 30 nl should reach a slightly greater spread of about 324 μm. Accordingly, when we moved the pipette tip 300 μm or more from the site where expiratory activity was encountered, the respiratory response to DLH microinjections failed to occur. It is also very unlikely that DLH-induced response resulted from non-specific effects of volume or pressure, since injecting equal or even larger volumes of saline or artificial cerebrospinal fluid did not alter respiratory activity.

Microinjections of excitatory amino acids have been proved to restrict the stimulation to the cell soma without activating axons of passage (Zieglgansberger & Puil, 1973; Goodchild *et al.* 1982). However, criticism has been raised against the interpretation of results from chemical stimulation based on the simple assumption that the responses are due to the excitation of neurones at the pipette tip (Lipski, Bellingham, West & Pilowsky, 1988). The main point concerns the observation that pressure injection of 5–150 nmol (10–150 nl of 0·5–1·0 M) of excitatory amino acids can produce a depolarization block of neurones in the immediate vicinity of the pipette tip after initial neuronal excitation. Thus, the responses may result from an inhibition rather than activation of neurones. In accordance with the suggestion of Lipski *et al.* (1988), in the present study we always injected DLH in amounts < 5 nmol in volumes usually < 30 nl to minimize the establishment of concentrations which could produce local depolarizing blocks. In any case, only the effects immediately following the injections were considered in the interpretation of results, to avoid possible misleading effects due to the development of depolarization blocks. Furthermore, the respiratory response elicited by DLH had both inhibitory and excitatory components and it seems unlikely that a depolarization block of neurones in this area could produce such a complex response. Finally, even if we admit the possibility of a small area of depolarization block at the centre of the injected area, the excitation of a large number of caudal medullary expiratory neurones is well proved by the powerful activation of the expiratory motor output.

The possibility exists that not only bulbospinal expiratory neurones, which are densely packed in the caudal VRG and

seem to be the most representative group of neurones in this region (e.g. Merril, 1970, 1974; Arita *et al.* 1987; for reviews see Long & Duffin, 1986; von Euler, 1986), but also other types of neurones located there may be affected by chemical stimulation. Silent neurones as well as neurones with either non-respiratory or respiratory discharge patterns, such as propriobulbar expiratory neurones or early-inspiratory neurones (Arita *et al.* 1987) may lie intermingled with expiratory bulbospinal neurones in the caudal VRG. An involvement of these other types of neurones cannot, at present, be completely ruled out. However, expiratory bulbospinal neurones are, in our opinion, the most likely candidates for the mediation of the observed effects.

Support for the present findings is provided by the results from recent neuroanatomical studies (Smith, Morrison, Ellenberger, Otto & Feldman, 1989) showing extensive ipsi- and contralateral interconnections between medullary respiratory neurones; projections from the caudal VRG to other medullary respiratory areas may constitute the anatomical substrate which mediates the effects observed in the present investigation.

The results we obtained in lignocaine blockade or transection experiments clearly indicate that medullary mechanisms subserve DLH-induced effects. In contrast to previous electrophysiological (Merrill 1974, 1979; Lipski *et al.* 1984) and neuroanatomical findings (Kreuter *et al.* 1977; Kalia, 1981; Arita *et al.* 1987), these results support the view that caudal medullary expiratory neurones have axon collaterals and appropriate connections with other medullary or upper cervical cord respiratory neurones to exert their infuence on central respiratory activity (Figs 6 and 7). The discrepancy between present and previous results could be attributed to methodological differences; for example, previous negative results do not preclude the existence of collaterals which are shorter than 0·5 mm (Mulloney & Selverston, 1972; Merrill, 1974) or collaterals arising at a considerable distance away from the cell body (Kreuter *et al.* 1977). Furthermore, it should also be taken into account that the population of expiratory neurones in the caudal VRG is far from homogeneous, both from the morphological and the functional point of view (see e.g. Lipski *et al.* 1984; Arita *et al.* 1987; Smith *et al.* 1989). Axon collaterals could either be relatively rare or characteristic of only some subgroups of neurones so that they provide very weak interactions, if any, during normal eucapnic breathing, but may reveal their whole efficacy when intense or massive activation of the parent neurones occurs.

From this point of view, present data do not completely refute the conclusion drawn from transection or lesion experiments (Merrill, 1979; Huang & St John, 1988; Speck & Beck, 1989), i.e. that caudal expiratory neurones are not essential for respiratory rhythm generation. Rather, they support the possibility that these neurones can have a role in altering or shaping the pattern of breathing when strongly activated. This could be relevant to some physiological conditions, such as reflex acts like the cough or the expiration reflex (for a review see Widdicombe, 1986), which require the activation of expiratory motoneurones and the concomitant inhibition of inspiratory activity, or increased central chemoceptive drive (Bainton & Kirkwood, 1979; St John, Bartlett, Knuth & Hwang, 1981). It is worth noting that DLH-induced respiratory effects were not accompanied by significant changes in arterial blood pressure, as expected from the results of lesion experiments (Speck & Beck, 1989). However, lesions affect not only cell bodies but also axons of passage, which may have a role in cardiovascular control.

The response of expiratory neurones of the caudal VRG contralateral to the injection site (Fig. 4) provides further insights into the neural pathways involved in the mediation of the bilateral excitation of expiratory motoneurones in response to unilateral stimuli applied to the caudal VRG. Since virtually all caudal medullary expiratory neurones have projections to the contralateral spinal cord (Merrill, 1970, 1974; for reviews see Long & Duffin, 1986; von Euler, 1986), bilateral activation of expiratory motoneurones can be achieved (apart from the presence of few, if any, uncrossed ipsilateral axons) via collaterals of crossed axons that recross at the level of the spinal cord probably near termination sites (Merrill, 1974; Cohen, Feldman & Sommer, 1985; Miller, Tan & Lakos, 1989). Present results provide direct evidence of a new pathway, most probably through axon collaterals of caudal medullary neurones providing excitatory inputs to their contralateral counterpart.

The responses observed in the rostral expiratory neurones are difficult to interpret. They recall the behaviour of these neurones during active expirations (sneezing) induced in freely moving cats (Orem & Brooks, 1986). Reciprocal connections recently shown between the Bötzinger complex and the caudal VRG (Smith *et al.* 1989) may subserve inhibitory interactions between caudal and rostral medullary expiratory neurones.

A final point to be discussed briefly is the strong depression or complete suppression of postinspiratory activity in the early recovery breaths following DLH-induced inhibition in phrenic nerve activity (Fig. 3). A progressive shortening of postinspiratory activity, associated with corresponding increases in the activity of expiratory muscles, has been found to be induced by hypercapnia in the dog (Oliven, Deal, Kelsen & Cherniack, 1985). A shortening of postinspiratory activity during hypercapnia has also been observed in the cat (see Prabhakar, Mitra, Overholt & Cherniack, 1986 and references therein). Depressant effects on postinspiratory activity could be mediated at the medullary level; reciprocal inhibitory mechanisms preventing simultaneous occurence of postinspiratory activity and expiratory muscle activities have been suggested (Richter, 1982; for reviews see Long & Duffin, 1986; von Euler, 1986). However, present results do not exclude the possibility that these effects are determined, at least in part, at spinal levels, where interneurones with inspiratory, expiratory and postinspiratory patterns of discharge have been found (Kirkwood *et al.* 1988; Bellingham & Lipski, 1990).

In conclusion, we propose that caudal medullary expiratory neurones may affect the pattern of breathing probably via medullary axon collaterals which interact

extensively with the respiratory neuronal network located in the medulla oblongata and upper cervical spinal cord. In contrast with previous evidence, data presented here support the hypothesis that these neurones are not merely upper motoneurones conveying the expiratory drive to the spinal cord.

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