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NEURONES IN COMMISSURAL NUCLEUS TRACTUS SOLITARII REQUIRED FOR FULL EXPRESSION OF THE PULMONARY C FIBRE REFLEX IN RAT

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SUMMARY

1. The pulmonary C fibre reflex, triggered by activating pulmonary C fibre endings in the lung, consists of rapid shallow breathing (which may be preceded by apnoea), bradycardia, and hypotension. The purpose of this work was to identify proximal synapses in this reflex. From pilot data, we hypothesized that neurones in a discrete region of the commissural nucleus in the nucleus tractus solitarii (NTS) are required for full expression of the pulmonary C fibre reflex. Studies were carried out in urethane-anaesthetized, unilaterally vagotomized, spontaneously breathing rats, in which diaphragm electromyogram, arterial pressure, and blood gases were measured. Phenyldiguanide (PDG) was injected in the right atrium to elicit the pulmonary C fibre reflex. Unilateral NTS injections were made through multibarrelled pipettes containing DL-homocysteic acid (DLH) to mimic the reflex, cobalt chloride to reversibly impair the reflex, and/or dye to mark the injection sites.

2. PDG (5–16 μg kg^{-1}) injected in the right atrium of twenty-six rats produced the classic pulmonary C fibre reflex: a vagally mediated, rapid onset of rapid shallow breathing, bradycardia and hypotension.

3. Injection of DLH (3–12 nl of 20 mM for a total of 60–240 pmol) in the dorsomedial aspect of the commissural nucleus of the NTS in thirty rats mimicked the pulmonary C fibre reflex, producing rapid shallow breathing, hypotension, and a slight bradycardia.

4. Interruption of neuronal transmission by injecting cobalt chloride (15–30 nl of 100 mM) in the site where DLH produced rapid shallow breathing, reversibly impaired the rapid shallow breathing and bradycardia produced by right atrial injections of PDG in fifteen rats. The commissural region where DLH produced rapid shallow breathing and cobalt impaired the pulmonary C fibre reflex extended from 720–1100 μm caudal to the obex, 30–200 μm lateral to mid-line, and 200–600 μm ventral to the dorsal surface of the brain stem within the NTS.

5. Taken together, the results suggest that neurones within a discrete region in the dorsomedial commissural nucleus in caudal NTS are required for full expression of the pulmonary C fibre reflex.

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INTRODUCTION

A wide-spread system of non-myelinated vagal C fibre afferents has been demonstrated in the lungs and airways (Coleridge & Coleridge, 1984). Two groups of these C fibre afferents have been distinguished by their location and vascular accessibility: the pulmonary C fibre afferents (juxtapulmonary or J receptors; Paintal, 1969) originating in lung parenchyma and supplied by the pulmonary circulation, and the bronchial C fibre afferents originating in the tracheobronchial walls and supplied by the systemic circulation (Coleridge, Coleridge & Luck, 1965; Kaufman, Coleridge, Coleridge & Baker, 1980).

The discharge properties of pulmonary C fibre afferents and the reflexes triggered by activating them have been studied largely by using chemical stimuli. While responsiveness varies somewhat by species, the afferents are most commonly stimulated by injecting phenyldiguanide, a serotonin structural analogue (Glogowska, Richardson, Widdicombe & Winning, 1972; Sapru, Willette & Krieger, 1981), or capsaicin (Coleridge *et al.* 1965) into the pulmonary circulation. Pulmonary C fibre endings also are activated by exposure to the environmental toxicants ozone (Lee, Dumont, Djokic, Menzel & Nadel, 1979) and cigarette smoke (Lee, Kou, Frazier, Beck, Pisarri, Coleridge & Coleridge, 1989) and during pulmonary venous congestion or oedema (Glogowska & Widdicombe, 1973; Coleridge & Coleridge, 1977; Hatridge, Haji, Perez-Padilla & Remmers, 1989). The reflex response consists of rapid shallow breathing (which may be preceded by apnoea), bradycardia and hypotension (Coleridge & Coleridge, 1984).

Although these reflex changes in respiratory motor output evoked by activating pulmonary C fibre endings and the potential role of this afferent system in certain pulmonary pathological states have been studied extensively, the central neural pathway responsible for transmitting the afferent input has not been described. The non-myelinated pulmonary C fibre afferents course in the vagus and would be expected to terminate in the nucleus tractus solitarii (NTS), where other respiratory sensory inputs are organized (Kalia & Mesulam, 1980). In a recent report in cats, Kubin, Kimura & Davis (1991) localized central axonal projections of broncho-pulmonary C fibre afferents to the commissural nucleus of the NTS caudal to the obex and in the parvicelluar nucleus of the NTS rostral to the obex. Still we do not know the location of neurones which are required for full expression of the pulmonary C fibre reflex. In preliminary studies, we identified a discrete region in the dorsomedial aspect of commissural nucleus of the NTS where direct chemical stimulation of neurones produced rapid shallow breathing, hypotension, and a small bradycardia. We hypothesized that this caudomedial NTS region contains neurones that receive pulmonary C fibre afferent input and are required for producing the pulmonary C fibre-activated reflex. If the hypothesis is true, then (1) direct chemical stimulation of a sufficient number of these NTS neurones should mimic the rapid shallow breathing evoked reflexly by activating pulmonary C fibre endings and (2) local interruption of neuronal activity in this NTS region should impair the reflex response to activating pulmonary C fibre endings. The specific aims of this study were: (1) to establish that phenyldiguanide (PDG) injected in the right atrium in rats elicits the classic, vagally mediated pulmonary C fibre-evoked reflex consisting of

rapid shallow breathing, hypotension and bradycardia; (2) to characterize further the site in the caudomedial NTS where local discrete injections ($\leqslant 12$ nl) of the excitatory amino acid DL-homocysteic acid (DLH) mimics pulmonary C fibre-evoked rapid shallow breathing; and (3) to determine if local interruption of neuronal transmission in this NTS region by cobalt chloride impairs the pulmonary C fibre-evoked rapid shallow breathing.

METHODS

General animal preparation

Experiments were performed on male Sprague–Dawley rats (300–400 g) anaesthetized with urethane (1200 mg kg^{-1} I.P.) and given supplemental doses (60 mg kg^{-1} I.P.) as needed. We assessed the adequacy of anaesthesia approximately every 30 min by testing for the absence of a withdrawal reflex, an increase in systemic arterial pressure, or an increase in breathing rate in response to paw pinch.

Diaphragm electromyograms (EMG), arterial pressure, heart rate, and blood gases were measured in every animal. For EMG recording, the diaphragm was exposed through an incision parallel to the caudal edge of the rib-cage. Two Teflon-coated silver wires (outer diameter 0·10 mm) were stripped 2 mm at each end, inserted into the diaphragm, and connected to a high-impedance follower. A catheter was inserted into the carotid or femoral artery and advanced to the thoracic or abdominal aorta to record arterial pressure, derive heart rate, and monitor arterial blood gases. A second catheter of pre-measured length was advanced through the right external jugular vein into the right atrium for injections into the pulmonary circulation. We confirmed right atrial catheter position post-mortem. Raw and integrated (20 ms time constant) diaphragm EMG and blood pressure were fed in parallel to an oscilloscope, polygraph, and tape-recorder for data analyses. Each rat was prepared with an endotracheal tube and allowed to spontaneously breathe either room air or oxygen-enriched room air to main oxygen saturation $> 95\%$. To restrict pulmonary C fibre afferent input to one side of the brain, we cut the cervical vagus contralateral to the NTS injection site. Body temperature was maintained with a servo-controlled water blanket. The rat's head was placed in a stereotaxic frame, with the head ventroflexed so that the dorsal brain stem was horizontal. An occipital craniotomy was performed and the caudal portion of the fourth ventricle was exposed by removing the dura and arachnoid membranes.

Pulmonary C fibre activation

To evoke the pulmonary C fibre reflex, we injected phenyldiguanide (PDG) into the right atrium in thirty unilaterally vagotomized rats. PDG was administered in doses of 5–16 μg kg^{-1} in 100 μl volumes over 2–3 s. Since tachyphylaxis to PDG has been shown to occur (Coleridge & Coleridge, 1984), we injected it (10 μg kg^{-1}) in two rats at least four times at each of three time intervals (15, 30 and 40 min). Tachyphylaxis did not occur with 40 min intervals, so all subsequent studies used a minimum of 40 min between PDG injections. To verify that the respiratory and circulatory responses to PDG injected in the right atrium were vagally mediated, it was injected after bilateral cervical vagotomy in a subset of nine rats.

To determine whether PDG changed respiratory motor output when administered into the systemic circulation, we injected it in the thoracic aorta of two rats. PDG doses (10 and 16 μg kg^{-1}) that produced rapid shallow breathing, bradycardia and hypotension when injected in the right atrium (pulmonary circulation) had no effect when injected in the thoracic aorta (systemic circulation) in the same rat. As a volume control, 100 μl of normal saline was also injected into the right atrium in two rats and had no effect on respiratory pattern, heart rate or arterial pressure.

Injection of DL-homocysteic acid in the NTS

To identify sites in the NTS where increasing neuronal activity mimicked the pulmonary C fibre-evoked rapid shallow breathing, we injected the broad-spectrum excitatory amino acid agonist DL-homocysteic acid (DLH; 3–12 nl; 20 mM, 60–240 pmol) in thirty-two rats. Injections were made with a pressure injection system through multibarrelled pipettes (tip diameter 5–15 μm). One barrel contained DLH and a second barrel contained 2% Chicago Sky Blue to mark the injection site(s). Polyethylene tubing connected each barrel to a regulated, solenoid valve-controlled

pressure source. The volume of each injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a compound microscope equipped with a fine reticle. Using a dissecting microscope, we placed the tip of the pipette on the dorsal surface of the brain stem at the level of calamus scriptorius; the stereotaxic co-ordinates of this point defined a relative zero for rostrocaudal, dorsoventral, and lateral movements of the pipette. Based on data from pilot studies, we searched the NTS from 100 μm rostral to 500 μm caudal to calamus scriptorius, from mid-line to 200 μm lateral to mid-line, and from 200–700 μm ventral to the dorsal surface. DLH was injected in volumes of 6–12 nl at 100–200 μm dorsoventral increments in each track. From these searches, we located the sites where the smallest doses of DLH produced the maximal duration of rapid shallow breathing. The histologically verified sites are described by their location with respect to the obex.

Injection of cobalt in the NTS region where DLH produced rapid shallow breathing

To determine if neurones in the site where DLH produced rapid shallow breathing were required for full expression of the pulmonary C fibre-evoked reflex, we injected cobalt chloride (15–30 nl; 100 mM) to temporarily interrupt neuronal activity in fifteen unilaterally vagotomized rats. The baseline pulmonary C fibre-evoked response was established by injecting PDG into the right atrium at least twice. Next we screened the caudomedial NTS for the site where the smalles dose of DLH produced rapid shallow breathing of the longest duration. This usually required one or two pipette penetrations. Then, without moving the pipette, we injected cobalt chloride through an adjacent barrel and repeated the PDG injection 10 min later. To assess recovery of the response from cobalt, PDG was then repeated every 40 min for up to 120 min. The site where DLH produced rapid shallow breathing and cobalt impaired the pulmonary C fibre-activated rapid shallow breathing was marked by injecting dye.

The extent of the NTS region required for expression of PDG-evoked rapid shallow breathing was functionally determined in a subset of nine rats. In these rats, after recovery of the PDG-evoked response from cobalt, we moved the pipette either 500–700 μm laterally ($n = 5$) or 500 μm rostrally ($n = 4$), repeated the cobalt injection, and 10 min later repeated the right atrial PDG injection. In these experiments, dye was injected in the nearby site.

Histology

At the end of each experiment, the brain was removed and fixed in 4% paraformaldehyde and 10% sucrose. Coronal sections, 40 μm thick, were counterstained with Neutral Red. The NTS sites where DLH produced rapid shallow breathing and cobalt impaired the PDG-evoked rapid shallow breathing were reconstructed from the dye spots and pipette tracks.

Data presentation and analyses

The respiratory responses to DLH and PDG were quantified by duration of rapid shallow breathing, maximal increase in respiratory rate, and the time required for respiratory rate to return to baseline. We defined respiratory pattern as shallow if the height of the integrated diaphragm EMG was $\leqslant 70\%$ of the height of the integrated diaphragm EMG observed immediately before the onset of the response. The duration of rapid shallow breathing was defined as the time from the onset of the response to the time when two consecutive diaphragm EMG signals were $> 70\%$ of the height of the diaphragm EMG immediately before the onset of the response. The maximum increase in respiratory rate was determined by counting breaths for 5 s after the onset of the response and was expressed as percentage increase over baseline. The time for respiratory rate to return to baseline was defined as the time from the onset of the response to the time when breathing rate returned to 110% of baseline. We chose 110% of baseline because breathing rate could normally vary up to 10%. The circulatory responses to DLH or PDG were quantified by the peak decreases in heart rate and arterial pressure.

Respiratory and cardiovascular responses before and after PDG was administered in the right atrium or DLH was injected in the NTS were compared using a paired t test. PDG-evoked respiratory and cardiovascular responses after one and then both vagi were cut were also compared using a paired t test.

In the cobalt experiments, PDG-evoked respiratory and circulatory responses were compared before, during, and after recovery from cobalt injections in the DLH-responsive site by using an ANOVA (analysis of variance) for repeated measures; ANOVA was followed by Sheffe's test when appropriate to determine significant differences between means. In the subset of animals in which

cobalt also was injected outside the DLH-responsive site, the subsequent PDG-evoked respiratory and cardiovascular responses were compared to those evoked after recovery from cobalt injections in the DLH-responsive site by using a paired t test.

To determine whether disrupting neuronal activity in the DLH-responsive region altered resting respiratory and cardiovascular status, baseline respiratory rate, heart rate, and arterial pressure were compared before, during, and after recovery from cobalt injections by using ANOVA for repeated measures, followed by Sheffe's test when appropriate.

All values are presented as means ± standard error of the mean. We considered a probability of type I error $< 0{\cdot}05$ to be significant.

RESULTS

Pulmonary C fibre reflex

Control values for arterial blood gases and pH in unilaterally vagotomized rats were expressed as means ± standard deviation: pH = 7·42 ± 0·03, P_{O_2} = 86·1 ± 8·1 mmHg, O_2 saturation = 96·2 ± 1·2 %, and P_{CO_2} = 38·6 ± 3·6 mmHg.

PDG (5–16 μg kg^{-1}) injected in the right atrium in twenty-six of thirty unilaterally vagotomized rats produced an immediate onset ($\leqslant$ 1 s) pulmonary C fibre reflex response, i.e. rapid shallow breathing, bradycardia and hypotension. The PDG-evoked rapid shallow breathing lasted 6·8 ± 0·7 s. Repiratory rate increased by 220 ± 15 % ($P < 0{\cdot}001$) over a baseline rate of 92 ± 2·6 breaths min^{-1} and returned to baseline in 40 ± 6 s. PDG significantly decreased heart rate by 200 ± 15 beats min^{-1} ($P < 0{\cdot}001$) from a baseline rate of 397 ± 7·4 beats min^{-1}, and decreased mean arterial pressure by 30 ± 2 mmHg ($P < 0{\cdot}001$) from a resting pressure of 90 ± 3 mmHg. In the four remaining rats, PDG produced a slow irregular breathing pattern. Interestingly, in two of the four rats, injecting DLH in the NTS site typically associated with rapid shallow breathing elicited a bradypnoea.

As shown in Figs 1 and 2, the PDG-evoked respiratory and cardiovascular responses were vagally dependent. In the example shown in Fig. 1, PDG-evoked rapid shallow breathing (Fig. 1*A*) was abolished by bilateral vagotomy (Fig. 1*B*); the increase in respiratory rate was typically limited to two quick breaths, followed by a return to the slow baseline respiratory pattern characteristic of animals with no vagi. The bradycardia also was abolished, although a small depressor response remained. As shown in the summary data from nine rats (Fig. 2), bilateral vagotomy significantly attenuated the PDG-evoked duration of rapid shallow breathing (Fig. 2*A*), increase in respiratory rate (Fig. 2*B*), time required for respiratory rate to return to baseline (Fig. 2*C*), bradycardia (Fig. 2*D*), and hypotension (Fig. 2*E*). As expected from disrupting all vagal afferent and efferent activity, bilateral vagotomy slowed baseline respiratory rate from 86 ± 6·4 to 40 ± 3·3 breaths min^{-1} ($P < 0{\cdot}005$), increased resting heart rate from 385 ± 11 to 499 ± 15 beats min^{-1} ($P < 0{\cdot}001$), and increased resting mean arterial pressure from 90 ± 4·9 to 106 ± 6·6 mmHg ($P < 0{\cdot}03$).

Respiratory and cardiovascular responses to DLH injected in commissural NTS

DL-Homocysteic acid (DLH; 20 mM; 3–12 nl; 60–240 pmol) injected in the dorsomedial aspect of the commissural nucleus of the NTS in thirty rats produced rapid shallow breathing, hypotension and a slight bradycardia. The DLH-evoked rapid shallow breathing lasted 11 ± 1·1 s. Respiratory rate increased by 185 ± 20 % ($P < 0{\cdot}001$) over a baseline rate of 99 ± 4 breaths min^{-1} and returned to baseline rate in 43 ± 2·7 s. DLH produced a slight but statistically significant decrease in heart rate

of 18 ± 7 beats min^{-1} ($P < 0{\cdot}02$) from a baseline of $407 \pm 7{\cdot}7$ beats min^{-1} and a decrease in mean arterial pressure of 21 ± 3 mmHg ($P < 0{\cdot}001$) from a resting pressure of $93 \pm 3{\cdot}4$ mmHg.

Figure 3 shows a representative comparison of the changes in respiratory motor output, heart rate and arterial pressure produced by injecting PDG in the right

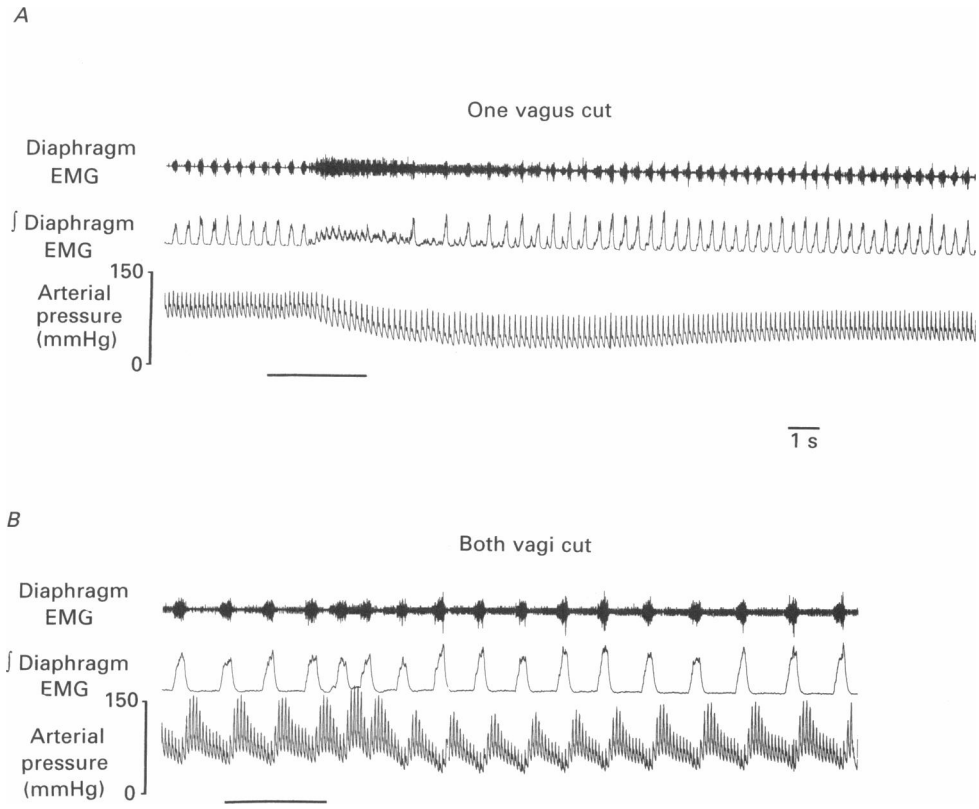

Fig. 1. Example of respiratory and cardiovascular responses to right atrial injection of PDG (10 $\mu g\ kg^{-1}$) with one vagus cut (*A*) and both vagi cut (*B*). Bar indicates injection.

atrium and injecting two doses of DLH in the NTS of the same rat. As shown in Fig. 3*A*, right atrial injection of PDG produced rapid shallow breathing, hypotension and bradycardia. DLH injected in a discrete region of the NTS in the same animal mimicked the PDG effect on respiratory pattern and arterial pressure in a dose-dependent manner (Fig. 3*B* and *C*).

The region from which DLH elicited the rapid shallow breathing was located in the caudomedial NTS and extended from 720–1100 μm caudal to the obex, from 30–200 μm lateral to mid-line, and from 200–600 μm ventral to the dorsal surface. Within individual rats, the boundaries circumscribing the region were distinct; that is, when the pipette was moved by 200–400 μm, the response to DLH was markedly reduced. Figure 4 shows the respiratory responses to DLH (12 nl; 20 mM) injected in three sites in one track through the commissural NTS. When the pipette was lowered to the dorsal border of the NTS (Fig. 4*A*), DLH produced a slight and delayed

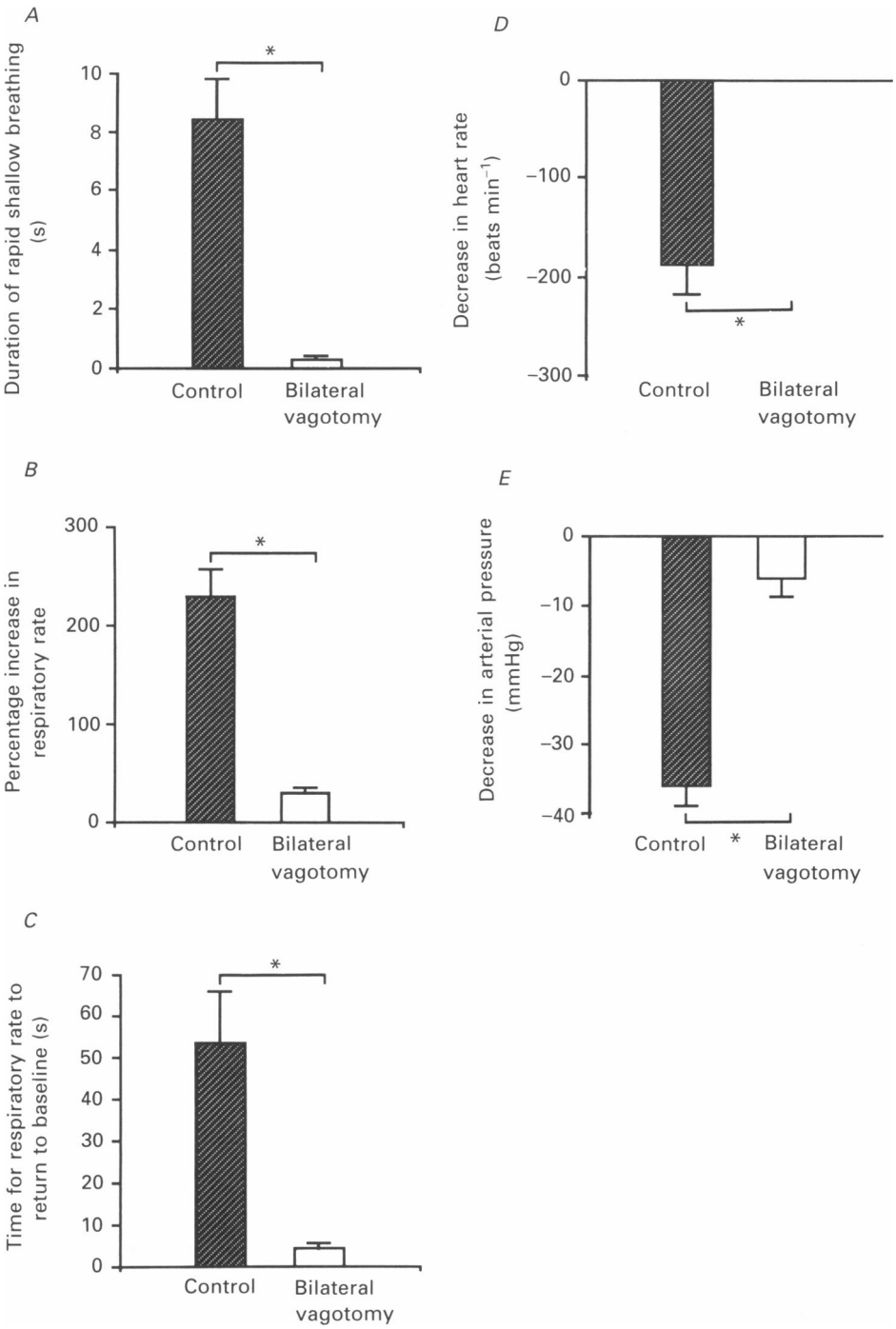

Fig. 2. Comparison of respiratory and cardiovascular responses to PDG injected in the right atrium in nine rats with one (Control) then both vagi cut (Bilateral vagotomy). * $P \leqslant 0{\cdot}005$; paired t test.

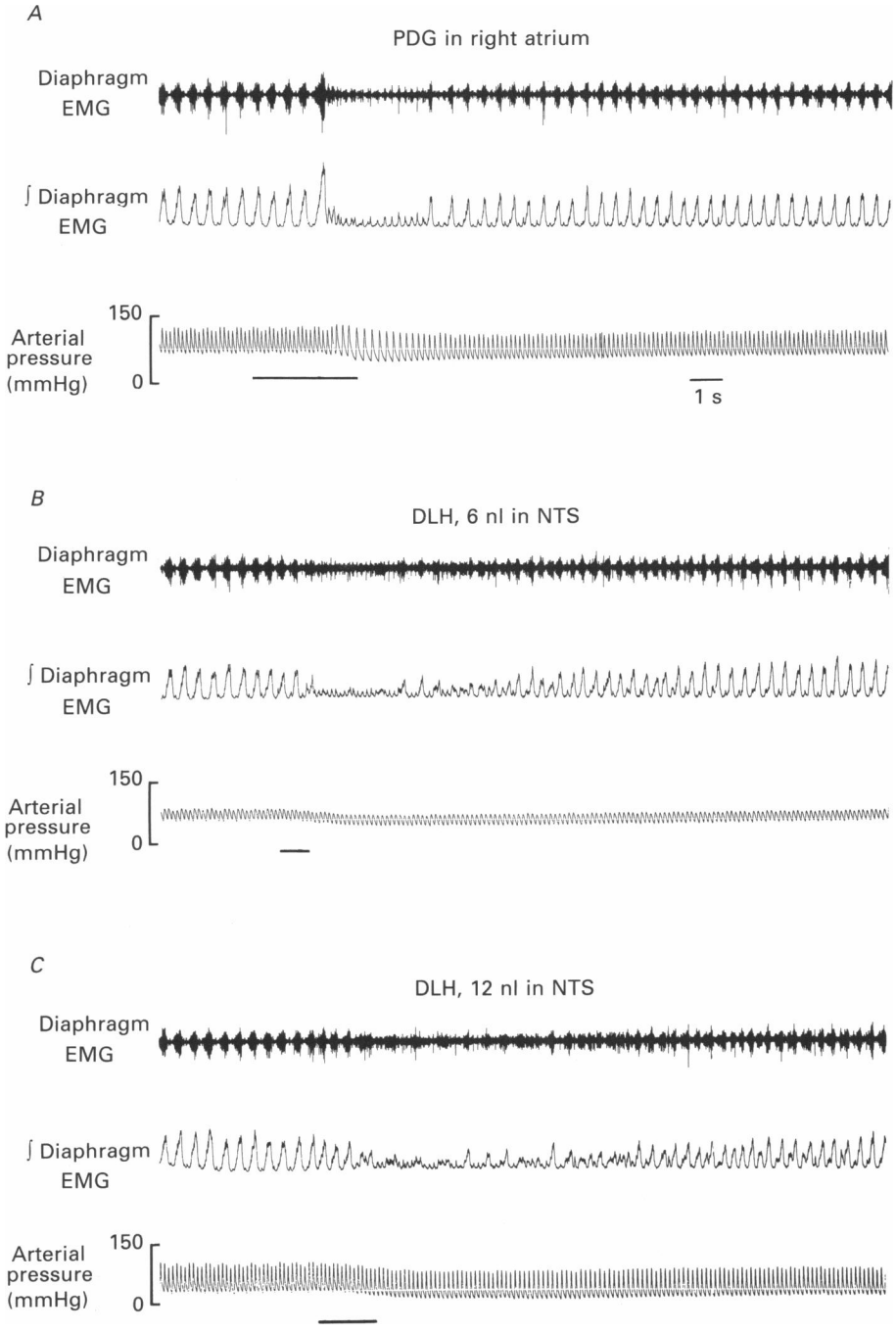

Fig. 3. Example of respiratory and cardiovascular responses to PDG (8 μg kg^{-1}) injected in the right atrium (*A*) and DLH, 6 nl (*B*) or 12 nl (*C*), injected in a discrete region of the NTS. Bar indicates injection.

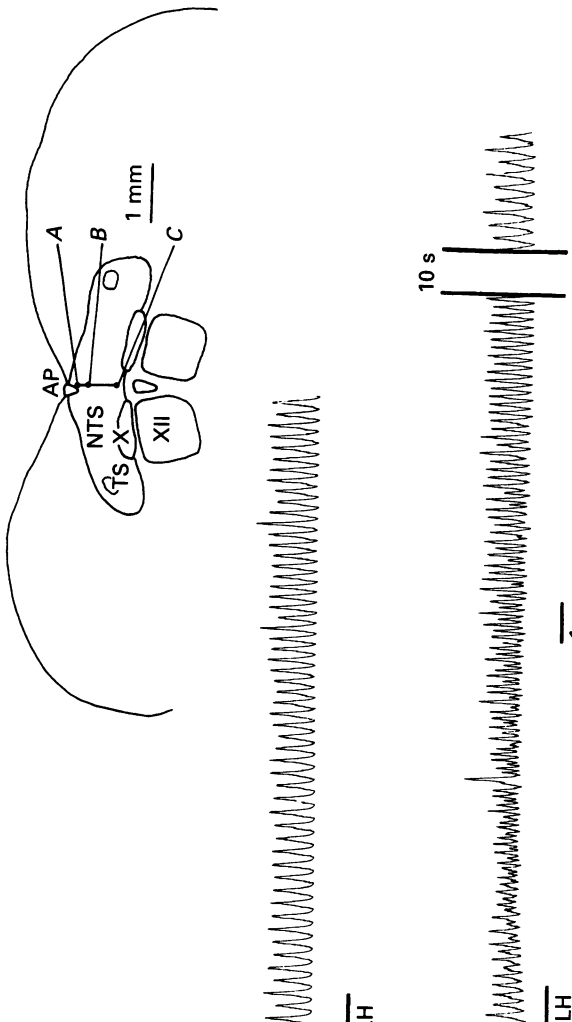

Fig. 4. Example of respiratory responses evoked by DLH (12 nl; 20 mM) at three sites in one track through the commissural nucleus of the NTS at 960 μm caudal to the obex and 50 μm lateral to mid-line: *A*, dorsal border of the commissural NTS; *B*, 200 μm ventral to *A*; *C*, 400 μm ventral to *B*. Bar indicates injection. AP, area postrema; TS, tractus solitarius; X, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus.

increase in respiratory rate. When the pipette was moved 200 μm ventrally (Fig. 4*B*), DLH produced an immediate-onset rapid shallow breathing. As the pipette was further displaced by 400 μm the response to DLH was gone (Fig. 4*C*).

Effect of cobalt in the DLH-responsive site on the pulmonary C fibre reflex

Cobalt (15–30 nl; 100 mM), injected in the NTS site where DLH (6–12 nl) produced rapid shallow breathing in fifteen unilaterally vagotomized rats, significantly

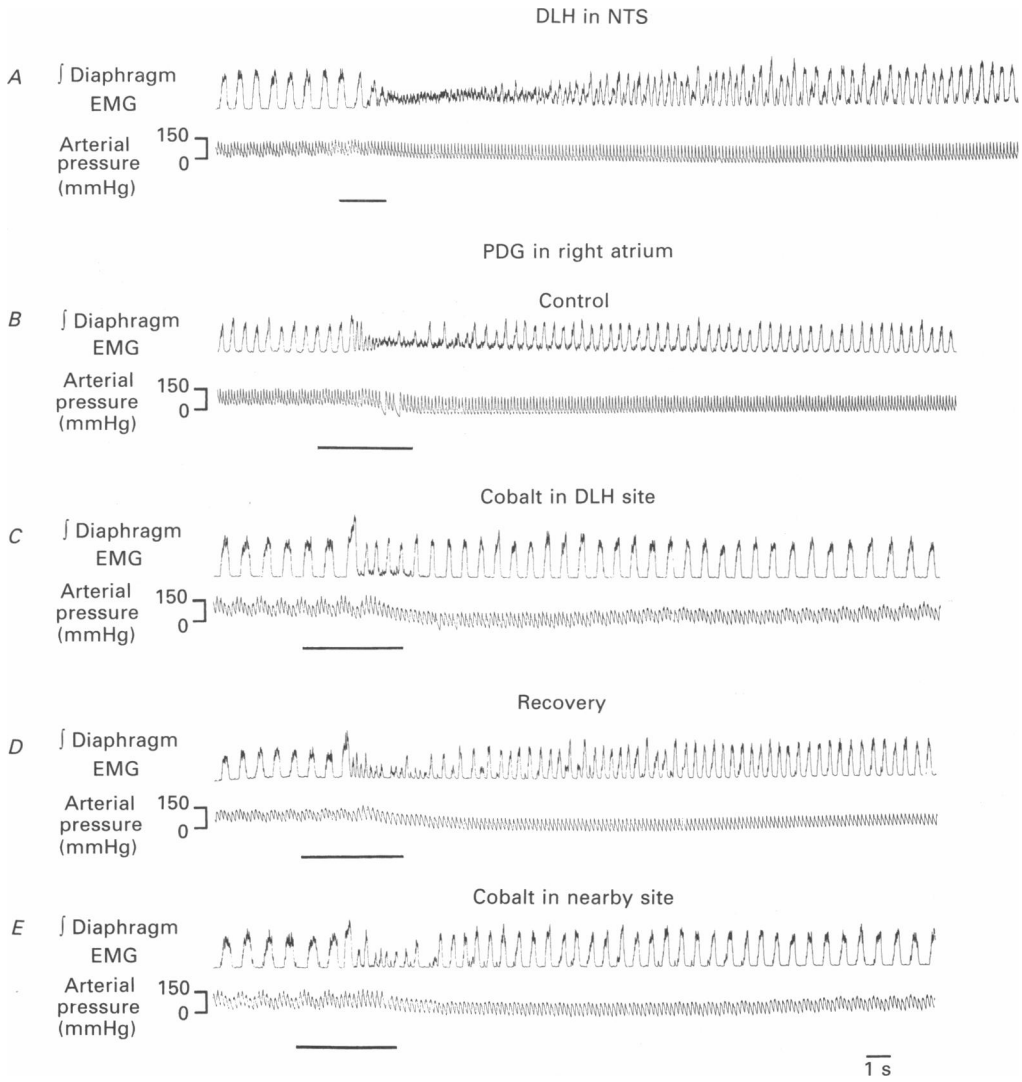

Fig. 5. Example of respiratory and cardiovascular responses to DLH injected in the NTS site (*A*), and PDG (8 μg kg^{-1}) injected in the right atrium under control conditions (*B*), 10 min after cobalt was injected in the DLH-responsive NTS site (*C*), 80 min after cobalt was injected in the DLH-responsive NTS site (*D*), and 10 min after cobalt was injected 600 μm lateral to the DLH-responsive NTS site (*E*). Bar indicates injection.

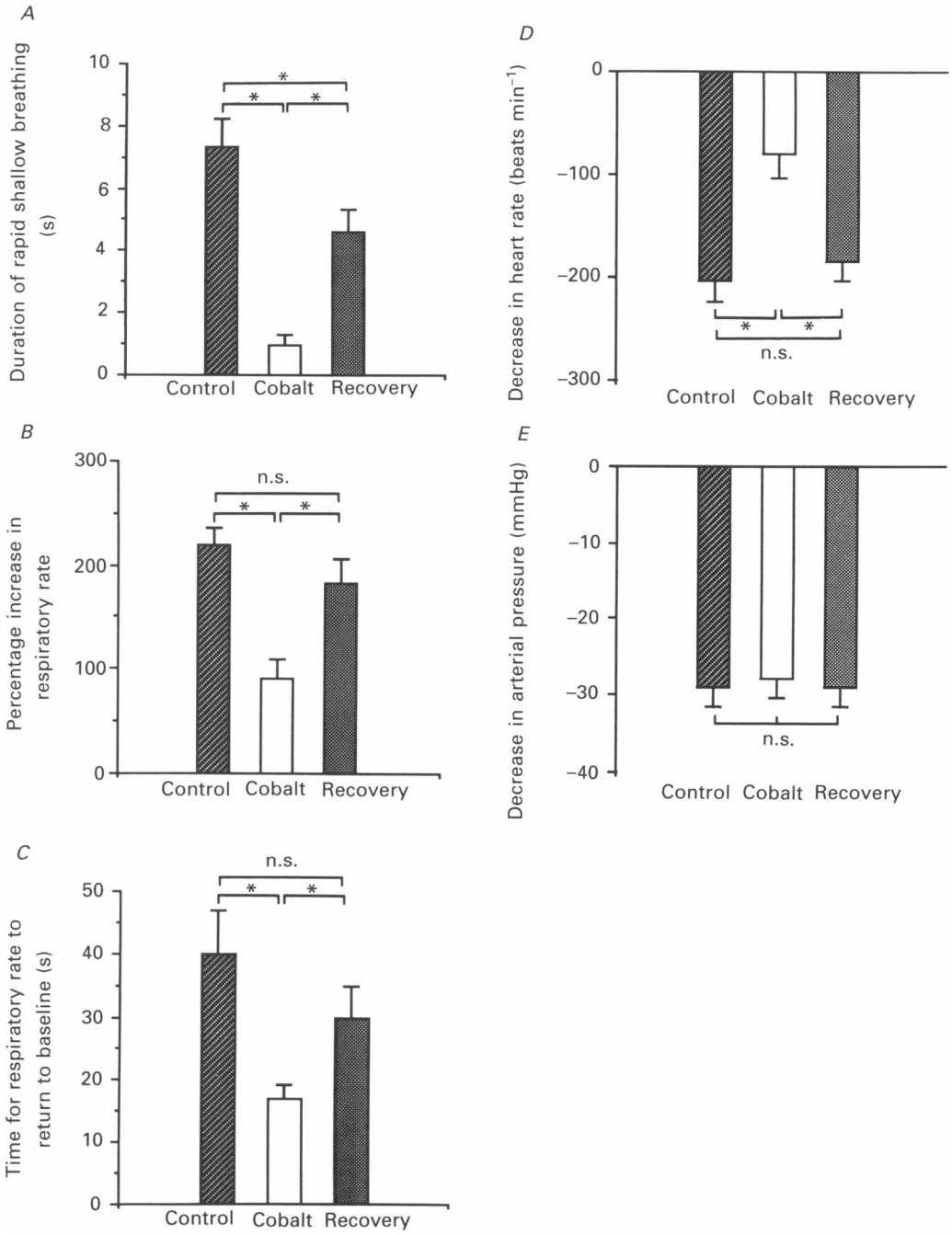

Fig. 6. Comparison of respiratory and cardiovascular responses to PDG in the right atrium in fifteen rats before (Control), during (Cobalt), and after (Recovery) cobalt (15–30 nl; 100 mM) was injected in the DLH-responsive site. $*P < 0{\cdot}005$, ANOVA; $P < 0{\cdot}05$, Sheffe's test.

attenuated the PDG-evoked rapid shallow breathing and bradycardia. In the example shown in Fig. 5, DLH (12 nl; 20 mM), injected in the NTS through one barrel of a three-barrel pipette, identified the optimal site where increasing neuronal activity mimicked the pulmonary C fibre-evoked rapid shallow breathing (Fig. 5*A*).

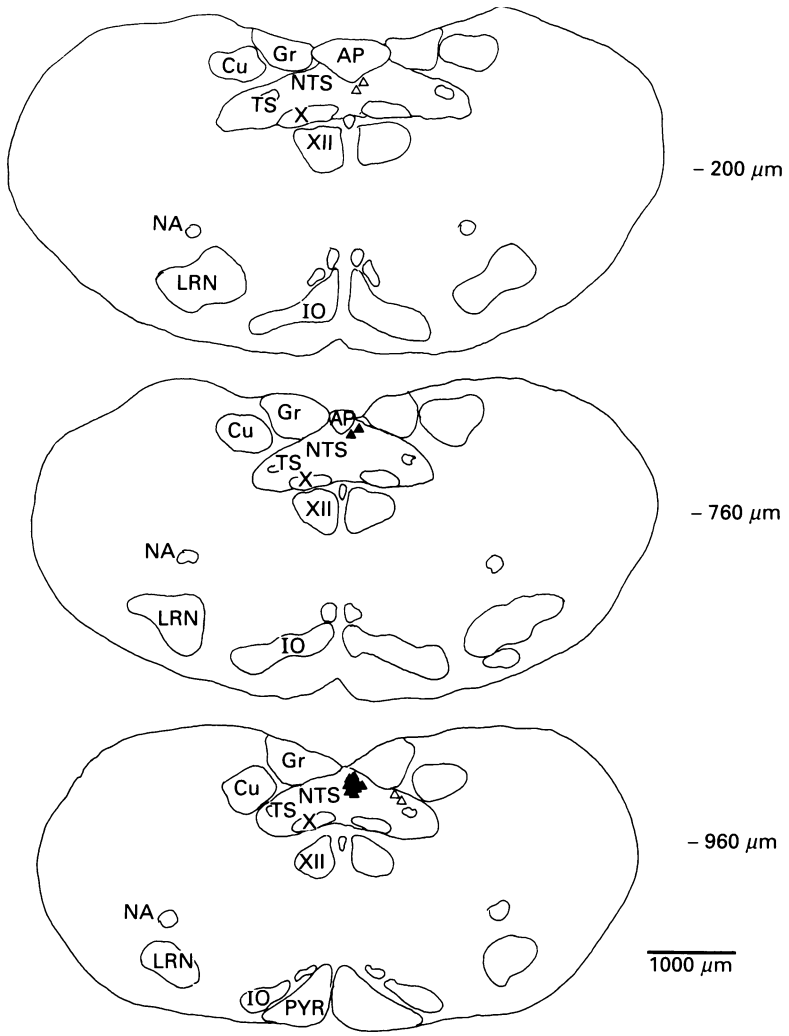

Fig. 7. Composite of histologically verified cobalt injection sites in thirteen rats. ▲, sites where cobalt injections in the DLH-responsive sites impaired the PDG-evoked rapid shallow breathing. △, sites where cobalt injections had no effect on the PDG-evoked responses. AP, area postrema; NTS, nucleus tractus solitarii; TS, tractus solitarii; Gr, gracilis nucleus; Cu, cuneate nucleus; X, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus; NA, nucleus ambiguus; LRN, lateral reticular nucleus; IO, inferior olivary nucleus; PYR, pyramids.

Right atrial injection of PDG (8 μg kg^{-1}) produced rapid shallow breathing, hypotension and bradycardia under control conditions (Fig. 5*B*). Cobalt, injected in the DLH-responsive site through the second barrel of the three-barrel pipette,

impaired the PDG-evoked rapid shallow breathing and bradycardia (Fig. 5*C*). The rapid shallow breathing response recovered from the cobalt in 80 min (Fig. 5*D*). After recovery, cobalt was then injected 600 μm lateral to the original site and did not further diminish the respiratory response to PDG (Fig. 5*E*).

As shown in the summary data (Fig. 6), locally interrupting neuronal transmission with cobalt significantly and reversibly attenuated the PDG-evoked duration of rapid shallow breathing (Fig. 6*A*), percentage increase in respiratory rate (Fig. 6*B*), and time required for respiratory rate to return to baseline (Fig. 6*C*). The duration of rapid shallow breathing partially recovered, while the increase in respiratory rate and time required for respiratory rate to return to baseline completely recovered. Cobalt also significantly and reversibly attenuated the PDG-evoked bradycardia (Fig. 6*D*), but did not alter the decrease in mean arterial pressure (Fig. 6*E*).

In addition to impairing the reflex effects of pulmonary C fibre activation, cobalt injected in the DLH-responsive site also slightly but statistically significantly changed baseline respiratory pattern and heart rate. In fifteen rats, baseline respiratory rate under control conditions was slowed by cobalt from 92 ± 3 to 79 ± 3 breaths min^{-1} ($P < 0{\cdot}05$) and recovered to 85 ± 3 breaths min^{-1}. Heart rate decreased slightly from 401 ± 14 to 351 ± 19 beats min^{-1} ($P < 0{\cdot}05$), and recovered to 397 ± 14 beats min^{-1}. Cobalt did not significantly change resting arterial pressure, which averaged 97 ± 4 mmHg under control conditions, 102 ± 4 mmHg in the presence of cobalt, and 95 ± 4 mmHg after recovery from cobalt.

To functionally identify the extent of the NTS region that was required for full expression of the pulmonary C fibre-activated reflex, cobalt was injected in nearby sites (500–700 μm lateral or 500 μm rostral to the DLH-responsive site) and did not alter the PDG-induced duration of rapid shallow breathing (5·6 *vs.* 5·2 s), increase in respiratory rate (220 *vs.* 230 %), time for respiratory rate to return to baseline (36 *vs.* 34 s), decrease in heart rate (180 *vs.* 170 beats min^{-1}), or decrease in mean arterial pressure (30 *vs.* 31 mmHg); all $P > 0{\cdot}1$.

A composite of the histologically verified sites where cobalt injected in the DLH-responsive site impaired the pulmonary C fibre-activated rapid shallow breathing is shown in Fig. 7. Filled triangles indicate sites where cobalt injections impaired the PDG-evoked rapid shallow breathing. The sites were concentrated in the dorsomedial commissural nucleus of the NTS in a region described by the following co-ordinates: from 720–1100 μm caudal to the obex, 30–200 μm lateral to mid-line, and 200–600 μm ventral to the dorsal surface of the brain stem within the NTS. Open triangles indicate sites where cobalt injections had no significant effect on the responses to right atrial injections of PDG.

DISCUSSION

Major findings

The major findings of this study were: (1) injections of phenyldiguanide (PDG) in the right atrium in unilaterally vagotomized rats produced rapid shallow breathing, hypotension and bradycardia, effects consistent with pulmonary C fibre activation; (2) 60–240 pmol of an excitatory amino acid injected in the dorsomedial commissural nucleus in the NTS also produced rapid shallow breathing, hypotension and bradycardia, mimicking the reflex responses triggered by PDG injected in the right

atrium; and (3) interruption of neuronal transmission in this DLH-responsive site reversibly impaired the rapid shallow breathing and bradycardia produced by PDG in the right atrium. These data suggest that neurones within this NTS region are part of the pulmonary C fibre afferent pathway and are required for full expression of the reflex responses.

Pulmonary C fibre evoked reflex

While PDG injected in the right atrium in rats in this study produced the classic reflex ventilatory response of rapid shallow breathing, an initial apnoea reported by some (Anand & Paintal, 1980; Sapru *et al.* 1981) but not all investigators (Karczewski & Widdicombe, 1969; Guz & Trenchard, 1971; Miserocchi, Trippenbach, Mazzarelli, Jaspar & Hazucha, 1978) was not seen. Although we did not observe an expiratory apnoea following right atrial injections of PDG, the respiratory rate during the rapid shallow breathing was quite fast, and sometimes approached a state of tonic diaphragmatic discharge, which may have resulted in a cessation of airflow. Indeed, this has been proposed by others (Badier, Jammes, Romero-Colomer & Lemerre, 1989; Hatridge *et al.* 1989) and suggests that in some cases the apnoea caused by pulmonary C fibre activation constitutes not an arrest of respiratory motoneuronal activity but rather a continuous activation of neural inspiratory activity such that movement of air ceases.

PDG is used extensively to stimulate pulmonary C fibre endings (Coleridge & Coleridge, 1984), but it is probably not a pure C fibre stimulant. Injected in the systemic circulation, PDG has been shown to stimulate carotid and aortic chemoreceptors in dogs (Comroe & Mortimer, 1964) and irritant receptors in cats and rabbits (Glogowska *et al.* 1972). Rats do not have functioning aortic chemoreceptors (Krieger & Marseillan, 1963) and stimulating their carotid bodies produces rapid eupnoeic breathing, tachycardia and hypotension (Lai, Tsuya & Hildebrandt, 1978; Housely & Sinclair, 1988) rather than the rapid shallow breathing, bradycardia and hypotension seen in this study, thus eliminating chemoreceptor stimulation as a major contributor to the observed responses. Stimulation of bronchial or other systemic C fibre endings also is unlikely to have confounded our results since the response onset to right atrial PDG was quite rapid and injection of PDG into the high thoracic aorta, in doses that produced rapid shallow breathing when injected in the right atrium, had no effect. There was a suggestion, however, that irritant receptors may have been weakly stimulated. Occasionally, augmented breaths were produced by PDG after cobalt was injected in the DLH-responsive site in the NTS, raising the possibility that blocking the neurones that receive pulmonary C fibre input may have unmasked the reflex production of an augmented breath by PDG (Fig. 5). PDG-induced augmented breaths have been ascribed to irritant receptor stimulation by Glogowska *et al.* (1972) and to both irritant and carotid chemoreceptor stimulation by Bartlett (1971). In our study, bilateral vagotomy abolished the augmented breaths, suggesting that irritant receptor rather than carotid chemoreceptor stimulation was responsible.

Establishing the NTS site for neurones in the pulmonary C fibre reflex pathway

This is the first report to localize NTS neurones in the pulmonary C fibre afferent pathway, by combining peripheral activation of pulmonary C fibre endings with

central injections of neuroactive agents to either mimic or impair production of the reflex responses. The triad of rapid shallow breathing, hypotension, and bradycardia produced by direct chemical stimulation of neurones in a discrete region of the NTS mimicked the response elicited by PDG injections in the right atrium. While mimicry of the ventilatory and cardiovascular responses to pulmonary C fibre activation suggests that those NTS neurones are part of the reflex pathway, certain issues must be considered in interpreting the data. (1) Were the responses caused by depolarization block rather than excitation of neurones in the immediate vicinity of the pipette tip? (2) Did the DLH spread beyond the immediate injection site? (3) Were the responses artifactual because of pressure and/or volume effects at the injection site? (4) Were the responses identical but unrelated to the activation of pulmonary C fibre endings?

Lipski, Bellingham, West & Pillowsky (1988) have shown that pressure injection of 5–15 nmol (10–150 nl of 0·5–1·0 M) of excitatory amino acids can produce depolarization block of neurones in the immediate vicinity of the pipette tip, prompting concern that responses may result from an inhibition rather than stimulation of neurones. In the present study, we injected 60–240 pmol of DLH (3–12 nl of 20 mM), at least 20-fold less than their lowest amount. Further, the response elicited by DLH was both excitatory (rapid shallow breathing) and inhibitory (bradycardia and hypotension), and it seems unlikely that depolarization block of neurones in this area could produce such a complex response.

In an attempt to restrict the spread of the injectate and hence the number of neurones excited, we injected small volumes (3–12 nl) of DLH. Theoretical calculations by Nicholson (1985) suggest that volumes of 10 nl should spread less than 300 μm from the injection site. Indeed, when we moved the pipette tip 300 μm in any direction, the response to DLH was greatly reduced or disappeared.

It is also unlikely that the responses to DLH resulted from non-specific effects of pressure or volume, since injecting equal or larger volumes of dye did not alter respiratory or cardiovascular status.

Finally, the evidence for linking neurones in the DLH-responsive site to the pulmonary C fibre afferent pathway becomes more compelling when local interruption of neuronal activity impairs the reflex triggered by activating pulmonary C fibre endings. Cobalt in low doses reversibly blocks synaptic transmission (Kretz, 1984), and in high doses can selectively destroy cell bodies while sparing fibres of passage (Lee & Malpeli, 1986). We have previously used cobalt to block transmission of the Breuer–Hering reflex in rats (Bonham & McCrimmon, 1990), and here to impair production of the pulmonary C fibre reflex. In the present study, cobalt injected into the DLH-responsive site reversibly impaired expression of the pulmonary C fibre reflex responses to right atrial injections of PDG; cobalt injections either 500 μm rostral or lateral did not significantly alter the reflex. Furthermore cobalt slowed baseline respiratory rate, suggesting that neurones in the region may contribute to eupnoeic breathing. Cobalt blockade of the pulmonary C fibre reflex was not complete, raising the possibility that the concentration or spread of cobalt was insufficient and/or that other obligatory pulmonary C fibre synapses were located elsewhere. Interestingly, cobalt did not block the hypotensive portion of the pulmonary C fibre reflex at all, suggesting that the cardiovascular haemodynamic component of the pathway may be organized elsewhere in the NTS.

The incomplete recovery of the response may have resulted from some neuronal destruction as described by Lee & Malpeli (1986).

In summary we have provided evidence that neurones in the dorsomedial region of the commissural nucleus of the NTS are required for the full expression of the pulmonary C fibre reflex. The site agrees well with one of two bronchopulmonary C fibre projection regions recently described in cats by Kubin, Kimura & Davies (1991). By electrically stimulating in the NTS to antidromically activate capsaicin-responsive cells in the nodose ganglion, they identified two central projection pathways for bronchopulmonary C fibre afferents: (1) a region caudal to the obex in the dorsal commissural nucleus and (2) a region rostral to the obex in the rim of the parvicellular subnucleus. The anatomical location of the site in the present study agrees strikingly with the caudal site described by Kubin & Davies (1989); however, whether a rostral projection site is present in rat is not known.

Functional organization of respiratory-related afferent input to the NTS

From data obtained by locally interrupting neuronal activity in the NTS, a picture is emerging of a functional organization of neurones required for producing respiratory and cardiovascular reflexes in rats. Using kainic acid lesions to impair the ventilatory response to hypoxia, Housely & Sinclair (1988) have recently demonstrated that an NTS region approximately 600 μm caudal to the obex, and lateral to the tractus (approximately 800 μm lateral to mid-line) contained neurones in the carotid chemoreceptor afferent pathway. Using cobalt injections to block lung inflation-induced apnoea, we have recently shown that a region, centred approximately 400 μm caudal to the obex and just medial to the tractus (approximately 600 μm lateral to mid-line), contained neurones required for producing the Breuer–Hering inspiratory-shortening reflex (Bonham & McCrimmon, 1990). In the present study, we provide data that neurones centred approximately 900 μm caudal to the obex and 100 μm lateral to the mid-line are required for production of the pulmonary C fibre reflex.

Together the data suggest that, at the level of the NTS, aggregates of neurones in the various respiratory afferent pathways are anatomically and functionally distinct. However, the final common pathway for all respiratory reflexes quite likely includes the respiratory premotor neurones in the dorsal respiratory group (DRG) and/or the ventral respiratory group (VRG). Therefore, it seems likely that interneurones located more distally in the reflex pathways, perhaps in the VRG or DRG, may represent sites for potential convergent inputs, either inhibitory or excitatory, from multiple respiratory sensory receptors.

In conclusion, this study is the first to provide evidence that neurones in the dorsomedial aspect of the commissural nucleus in the caudal NTS are part of the central pulmonary C fibre afferent pathway and are necessary for the full expression of the pulmonary C fibre reflex.

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