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RESEARCH ARTICLE

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The possible role of C5 segment inspiratory interneurons investigated by cross-correlation with phrenic motoneurons in decerebrate cats

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Abstract We tested the role of C5 segment inspiratory interneurons in transcribing central respiratory drive to phrenic motoneurons and mediating intersegmental reflexes by cross-correlating the spontaneous activity of 26 interneurons with that of the ipsi -and contralateral C5 phrenic nerves in decerebrate cats. There were 10 interneurons that discharged only during inspiration (phrenic burst) and 16 that discharged tonically with increased firing during inspiration. Of the cross-correlograms for 26 of the interneurons with the ipsilateral phrenic, 20 were flat and 2 had peaks centred about time zero, interpreted as a common activation of the interneurons and motoneurons. The cross-correlograms for 4 other interneurons had troughs centred about time zero, interpreted as a synchronous excitation of the interneurons and inhibition of the motoneurons. Of the cross-correlograms for 23 interneurons with the contralateral phrenic, 22 were flat and 1 had a peak centred about time zero, interpreted as a common activation of the interneuron and motoneurons. Nine of ten cross-correlograms between pairs of interneurons were flat; one had a peak centred about time zero. We conclude that, despite their inspiratory modulated discharge patterns, there is no evidence from this study that the C5 segment inspiratory interneurons convey central respiratory drive to C5 phrenic motoneurons.

Key words Respiration · Cross-correlation · Inspiratory interneurons · Phrenic motoneurons · Decerebrate cat

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Introduction

The diaphragm is driven to produce its respiratory motion by phrenic motoneurons in the spinal cord that receive descending inspiratory drive from two populations of neurons in the medulla, the dorsal and ventral respiratory groups (for reviews, see Bianchi et al. 1995; Ezure 1990). Controversy persists about the relative importance of monosynaptic versus oligosynaptic connections between medullary inspiratory bulbospinal neurons and phrenic motoneurons (for review, see Monteau and Hilaire 1991).

Both dorsal and ventral group inspiratory neurons have been shown using cross-correlation to drive phrenic motoneurons in the cat (Bianchi and Barillot 1974; Cohen et al. 1974; Davies et al. 1985a; Graham and Duffin 1982; Hilaire and Monteau 1976), and similar evidence has recently been obtained for the rat (Duffin and van Alphen 1995). Spike-triggered averaging experiments have reinforced the view that monosynaptic connections to phrenic motoneurons are common for inspiratory neurons of both the dorsal respiratory group (Fedorko et al. 1983; Monteau et al. 1985) and the ventral respiratory group with collaterals to the phrenic motor nucleus (Fedorko et al. 1989). This conclusion is consistent with neuroanatomical evidence in cat (Feldman et al. 1985), rabbit (Ellenberger et al. 1990) and rat (Dobbins and Feldman 1994; Ellenberger and Feldman 1988).

Although the existence of monosynaptic projections from medullary inspiratory neurons to phrenic motoneurons based on cross-correlation or spike-triggered averaging is therefore widely accepted, Davies et al. (1985a), using evidence from their cross-correlation studies and modelling calculations, argue that most of the depolarization of phrenic motoneurons is mediated via interneurons. Two pools of interneurons that may provide this function have so far been identified: the upper cervical inspiratory neurons and the interneurons associated with the phrenic motor nucleus.

The upper cervical inspiratory neurons form a column located near the lateral edge of the intermediate grey

matter in cats (Lipski and Duffin 1986) and rats (Lipski et al. 1993). In cats these neurons project mainly to intercostal motoneurons (Hoskin et al. 1988; Lipski and Duffin 1986), with a weaker projection to phrenic motoneurons (Douse et al. 1992; Lipski and Duffin 1986). In rats, intracellular labelling of upper cervical inspiratory neurons has shown that they project single short collaterals in the region of the phrenic nucleus (Lipski et al. 1993). While evidence for monosynaptic connections to phrenic motoneurons in cats has not been found using cross-correlation (Douse et al. 1992), evidence for monosynaptic or paucisynaptic connections has been obtained using averaging of phrenic nerve recordings in conjunction with focal cold block of descending bulbospinal respiratory pathways (Nakazono and Aoki 1994). In rats, while initial cross-correlation studies failed to disclose evidence for the synaptic connections to phrenic motoneurons suggested by the anatomical tracings (Lipski et al. 1993), more recent studies (Tian and Duffin, in press) indicate that such connections do exist.

Respiratory interneurons can be recorded (Baumgarten et al. 1963; Bellingham and Lipski 1990; Grélot et al. 1993 ;Palisses et al. 1989) in the region of the phrenic motor nucleus. Although interneurons in the C5 spinal segment with an expiratory discharge pattern have been shown using antidromic mapping to project to the C6 segment, and spike-triggered averaging experiments have demonstrated their connections to C6 phrenic motoneurons (Douse and Duffin 1993), the function and projection of interneurons with an inspiratory discharge pattern are unknown.

We tested the hypothesis that C5 inspiratory interneurons mediate, at least in part, the descending central respiratory drive to phrenic motoneurons. These neurons may also mediate intersegmental reflexes arising from thoracic afferents (for review see Shannon 1986). We tested these hypotheses by cross-correlating the activity of these interneurons with the activity of the ipsilateral and contralateral phrenic nerves.

Methods

Animal preparation

Experiments were conducted on nine female cats weighing 2.2–3.2 kg. After anaesthesia had been induced with an intravenous injection of a mixture of alfaxalone and alfadalone acetate (9 and 3 mg/kg, respectively; Saffan, Pitman-Moore), a tracheal cannula was inserted just below the larynx; anaesthesia was maintained with halothane in oxygen. A femoral vein and artery were cannulated to allow for the injection of drugs and monitoring of blood pressure (Harvard Apparatus). A surgical level of anaesthesia was maintained, characterized by a mean arterial blood pressure of around 100 mmHg and a lack of heart rate or blood pressure response to nociceptive stimuli. All procedures were reviewed and approved by the University of Toronto animal care committee.

The external carotid arteries were ligated. Both C5 branches of the phrenic nerves were isolated; the left one was cut and its central end desheathed in preparation for later recording. The right nerve was desheathed but otherwise left intact. The cat was then paralysed and ventilated to maintain end-tidal CO_2 at 4–6% (Datex); an expiratory threshold load of 3 cmH_2O prevented lung collapse. The cat was placed on its left side and the chest wall opened between the 6th and 7th ribs. The right intrathoracic phrenic nerve was isolated, freed from surrounding tissue, and the intact nerve placed inside the loops of a bipolar platinum electrode. The nerve-electrode combination was sealed inside a sheet of parafilm covered with petroleum jelly. The electrode lead was then led outside the chest wall and the incision closed.

After placement of the cat in a stereotactic frame (Kopf) and its suspension by means of hip pins and a clamp on the spinal process of the second thoracic vertebra, it was decerebrated. Halothane anaesthesia was then discontinued. Bilateral pneumothoraces were made to improve stability of recordings. The trunk of the cat was wrapped in a thermostatically controlled heating blanket to maintain the cat's rectal temperature at 37°C (Harvard Apparatus). A dorsal laminectomy from C2 to T1 exposed the dorsal surface of the cervical spinal cord. Agar was injected into the space between the spinal cord and the vertebrae and between the dura and the spinal cord after the dura had been split and retracted laterally. Warm agar was also poured over the spinal cord to a depth of several millimetres and, after it had hardened, a hole was cut in the agar to expose the surface of the spinal cord. Warmed mineral oil was poured over the surface, surface blood vessels over the fifth cervical segment were cauterized, and the pia over the sites of electrode placement removed.

Pools filled with warmed mineral oil were made for the phrenic nerves from the surrounding skin flaps. Both nerves were placed over bipolar silver electrodes and the activities amplified (Neurolog NL104), filtered (10 Hz to 10 kHz), and displayed on both an oscilloscope and a chart recorder as both raw and RC-integrated (Neurolog NL703; time constant 100 ms) signals. The signal from the right phrenic nerve was used to monitor both efferent activity and compound action potentials elicited by 1-ms shocks (usually about 2 V) at 10 times threshold to the supra-diaphragmatic phrenic nerve. Stimuli were obtained from an isolation unit (Digitimer DS2) driven by a stimulator (Digitimer D4030).

Recording

We recorded activity from respiratory interneurons in the right C5 cervical segment using glass-coated tungsten microelectrodes with impedances of approximately 250–500 kΩ at 1 kHz. The signals were amplified (Neurolog NL104) and filtered (bandpass 0.125–8 kHz). Interneurons were identified on the basis of rhythmic discharges related to phrenic activity and the absence of antidromic activation by suprathreshold (at least 10 times threshold) shocks applied to the ipsilateral phrenic nerve. Action potential waveforms and durations were those typical of cell bodies, not axons (Bellingham and Lipski 1990). Signals were displayed on oscilloscopes (Tektronics, Nicolet) and on a thermal array chart recorder (Graphtec WR3600), and stored on video tape in a digitized form (Vetter).

When an inspiratory interneuron was successfully isolated, we then attempted to isolate and record from a second inspiratory interneuron in the same segment, usually caudal to the first. Interneuron action potentials were discriminated using time-amplitude window gates (Bak), and the phrenic nerve potentials were discriminated using amplitude gates, the pulse outputs of which were fed to a purpose-built cross-correlator (Anderson and Duffin 1976). We used bin widths of 0.2 ms so that the phrenic nerve discrimination could be set to include even low-amplitude potentials without saturating the cross-correlator.

Analysis

The cross-correlation between the interneurons was usually computed on-line, the remaining cross-correlations (between both interneurons and both phrenic nerves) being computed off-line from the signals stored on video tape. Bin width was always 0.2 ms. Features observed in the cross-correlation histograms were quanti-

fied using the *k*-ratio (Sears and Stagg 1976). For peaks this was the ratio of the peak bin count to the mean bin count; for troughs it was the ratio of the sum of the mean count and the difference between the mean bin count and the nadir bin count to the mean bin count. The mean bin count was measured away from the features but also to avoid any bias effect of long-term trends (e.g. to the far right in the cross-correlograms shown in Fig. 3 and prior to time zero in Fig. 4). The features were then tested for statistical significance (Graham and Duffin 1981) at the P=0.001 level. For flat cross-correlograms the degree of interaction that may have been undetected was also calculated (Graham and Duffin 1981).

Values are expressed as mean±SD.

Results

Location and firing patterns of C5 inspiratory interneurons

The 26 inspiratory interneurons in this study were those successfully retained from a larger sample (n=52; 43 inspiratory, 8 expiratory, and 1 phase-spanning) of interneurons in the C5 cervical segment. These inspiratory interneurons were located between the mid-line and the dorsal root entry zone at depths between 2.5 and 4.0 mm (3.2±0.5), dorsomedial to the phrenic nucleus which was located, based on its maximum amplitude antidromic field potential, typically 4.0 mm from the dorsal surface of the spinal cord below the dorsal root entry zone.

Inspiratory interneurons discharged either tonically with an increased frequency of discharge during inspiration (16/26, 62%) or only during inspiration. An example of the discharges of a simultaneously recorded pair of interneurons and the ipsilateral (right) and contralateral phrenic nerves is depicted in Fig. 1. One interneuron was tonically active (Fig. 1A) with its discharge increasing during inspiration, whereas the other interneuron (Fig. 1B) was active only during inspiration.

Examples of autocorrelograms and interval histograms for these interneurons are shown in Fig. 2. The interspike intervals for some 65% of the interneurons were not strongly related, but instead suggested a random discharge pattern. The autocorrelograms for these neurons did not display a cyclic pattern (e.g. Fig. 2B,C). However, 35% of the interneuron autocorrelograms did show a regular pattern of peaks and troughs (e.g. Fig. 2A), indicating regular interspike intervals. For some tonically firing inspiratory interneurons, the interval histograms showed distinct firing frequencies for the expiratory and the inspiratory phases (e.g. Fig. 2A). The modal firing frequencies of the inspiratory interneurons was determined from the interval histograms and ranged from 14.5 to 74.6 impulses/s (30.4±13.0).

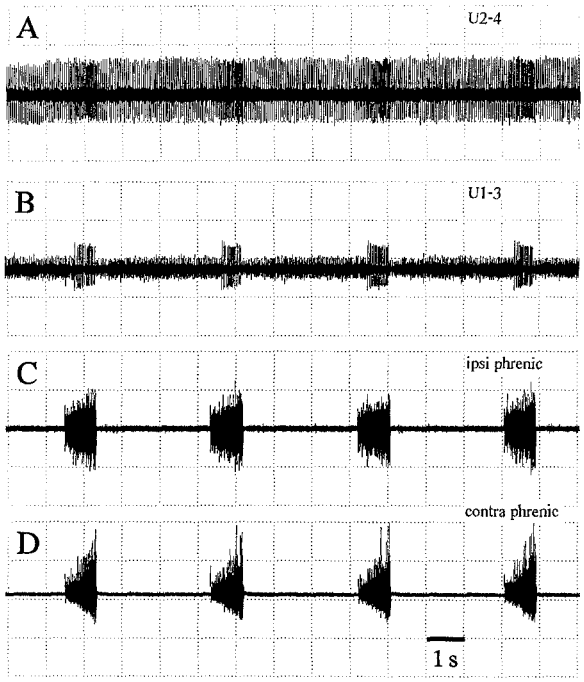

Fig. 1A–D Recording of a pair of C5 segment inspiratory interneurons. **A, B** Chart recordings of interneurons U2-4 (see also Fig. 2A) and U1-3 (see also Figs. 2B and 4D), respectively. **C, D** Discharge of the right (ipsi-phrenic) and left (contra-phrenic) phrenic nerves, respectively (ipsilateral and contralateral to the interneurons)

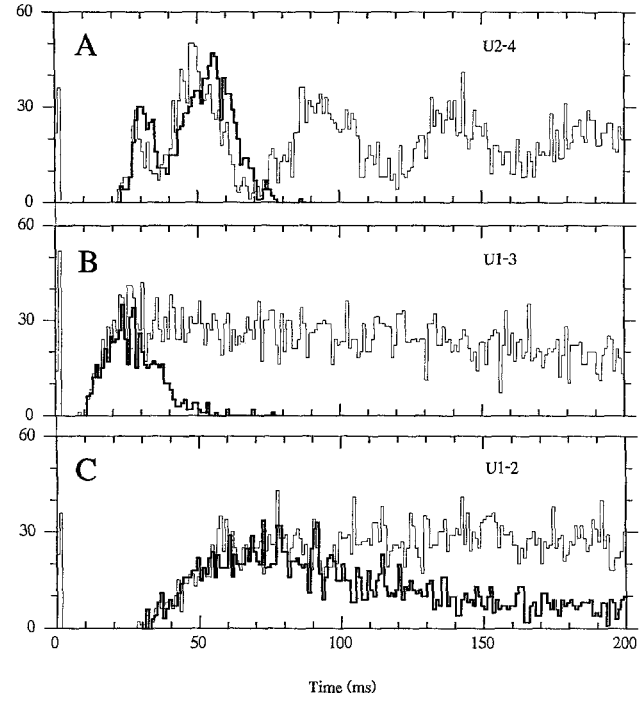

Fig. 2A–C Examples of autocorrelograms (*light traces*) and interval histograms (*heavy traces*) for C5 segment inspiratory interneurons. **A** Autocorrelogram and interval histogram for tonic interneuron U2-4 (see also Fig. 1A). The autocorrelogram displays cyclic features demonstrating the tendency of this interneuron to fire with regular interspike intervals. The interval histogram shows distinct firing frequencies during the expiratory (slower) and inspiratory phases. **B** Autocorrelogram and interval histogram for interneuron U1-3 (see also Figs. 1B and 4D). The autocorrelogram lacks cyclic features showing little evidence for regular interspike intervals. **C** Autocorrelogram and interval histogram for tonic interneuron U1-2 (Fig. 4A). The autocorrelogram lacks cyclic features and the interval histogram displays evidence of occasional long interspike intervals. Bin widths are 1 ms and the vertical axes are counts/bin

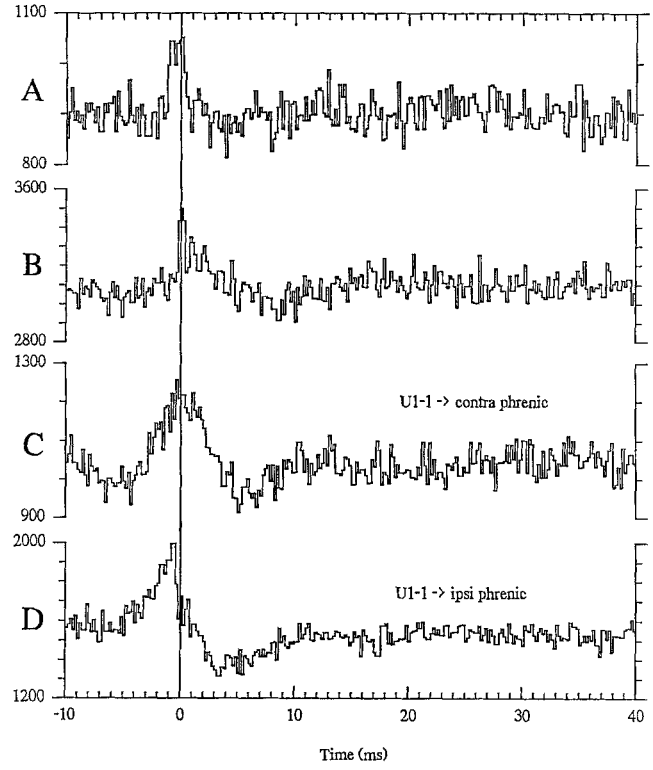

Fig. 3A–D Cross-correlograms displaying central peaks. **A** Between two C5 segment inspiratory interneurons. **B** Between a C5 segment inspiratory interneuron and the ipsilateral C5 phrenic nerve. **C, D** Between a C5 segment inspiratory interneuron and the contralateral (contra) and ipsilateral (ipsi) C5 phrenic nerves. Bin widths are 0.2 ms and the vertical axes are counts/bin

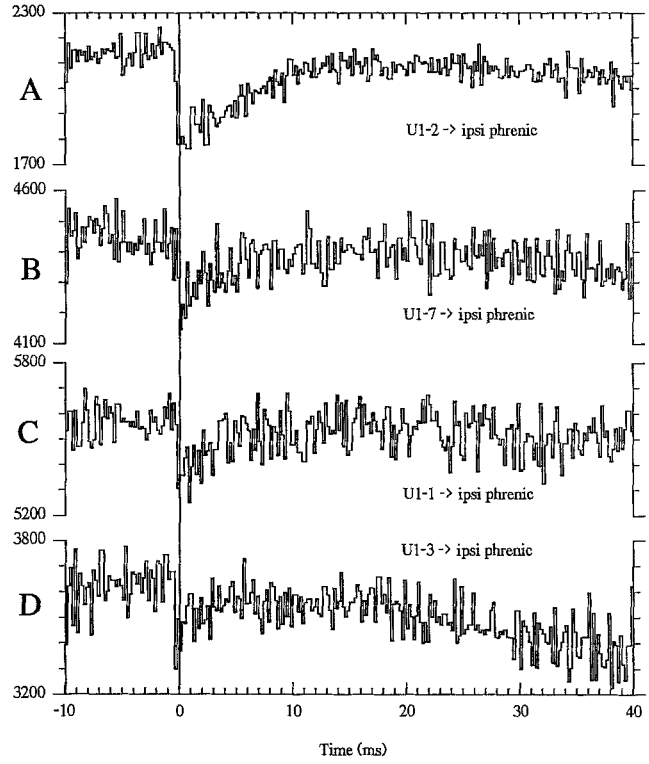

Fig. 4A–D Cross-correlograms displaying central troughs. All are between C5 segment inspiratory interneurons and the ipsilateral phrenic nerve. Cross-correlogram *D* is for U1-3 (Figs. 1B, 2B). Bin widths are 0.2 ms and the vertical axes are counts/bin

Connections from C5 inspiratory interneurons to phrenic motoneurons

The most common finding was a flat cross-correlogram between C5 inspiratory interneurons and ipsilateral (20/26) and contralateral (22/23) phrenic motoneurons.

The few significant features observed in the cross-correlograms were centred about time zero and consisted of two broad peaks, four broad troughs, and one combined peak and trough. One interneuron (Fig. 3C,D) had a broad peak in its cross-correlogram with the contralateral phrenic nerve discharge (half-amplitude width 4.4 ms) as well as a combined peak and trough in its cross-correlogram with the ipsilateral phrenic nerve discharge (half-amplitude width 2.0 ms). Another interneuron had a broad peak (half-amplitude width 2.4 ms) in its cross-correlogram with the ipsilateral phrenic nerve discharge (Fig. 3B). Four interneurons had troughs centred about time zero (half-amplitude widths 6.8, 3.2, 2.6 and 2.2 ms) in their cross-correlograms with the ipsilateral phrenic nerve discharge (Fig. 4A–D).

We estimated that the degree of interaction between interneurons and the ipsilateral and contralateral phrenic motoneurons that can have been missed was 4.4±2.5 and 4.3±1.2 events per 1000, respectively. This estimate is based on an assumed significance level of P=0.001 and an assumed mean firing frequency for phrenic motoneurons of 29 impulses/s in decerebrate cats (Milano et al. 1992).

Connections between C5 inspiratory interneurons

Of the ten cross-correlograms between interneurons, nine were flat and one had a peak with a half-amplitude width of 1.6 ms centred around the origin (Fig. 3A). These two interneurons were separated by 4.5 mm. The average separation between pairs of interneurons, based on surface landmarks, was 4.2±1.8 mm.

Discussion

Inspiratory interneurons were scattered in the C5 spinal segment dorsomedial to the phrenic motor nucleus, a location slightly medial to the expiratory interneurons described by Douse and Duffin (1993) and similar to that observed by Bellingham and Lipski (1990). Their typical firing patterns were tonic (62%), with an increase in firing frequency during inspiration and irregular intervals between action potentials (60%), similar to the thoracic inspiratory interneurons described by Kirkwood et al. (1988). They therefore differ from the bulbospinal inspiratory neurons of the medulla, which typically have ac-

tivity confined to the inspiratory phase and with regular intervals between action potentials (Graham and Duffin 1982; Long and Duffin 1984). However, their modal firing frequencies (30 impulses/s) were similar to those of bulbospinal inspiratory neurons of the medulla (Graham and Duffin 1982; Long and Duffin 1984).

The cross-correlograms between C5 inspiratory interneurons and phrenic motoneurons did not feature short-latency, narrow peaks. Instead, the cross-correlograms were characterized by either no peaks (42 of 49 cases for both ipsi- and contralateral phrenic nerves) or, less often, features centred about time zero for both the ipsi- and contralateral phrenic nerves, indicating a common synchronizing event. The central peaks were interpreted as evidence for shared excitation (e.g. Kirkwood and Sears 1991), although a shared inhibition could also have been responsible.

Central troughs were interpreted as evidence for a common synchronizing event that simultaneously excited the interneuron and inhibited the phrenic motoneurons (Moore et al. 1970), rather than an inhibitory connection from the interneuron to the phrenic motoneurons, because the troughs spanned time zero. The cross-correlogram shown in Fig. 3D appears to show a mixture of the two functions, phrenic inhibition and shared excitation.

Although the source of the common excitation cannot be determined from the cross-correlograms, it probably originates from either medullary inspiratory neurons or upper cervical inspiratory neurons. We also speculate that common excitation of these C5 inspiratory interneurons and C5 phrenic motoneurons and also the inhibition of C5 phrenic motoneurons result from inputs from other interneurons in the same and other (e.g. thoracic) spinal segments activated by descending inspiratory drive.

The overall results from the interneuron-to-phrenic cross-correlograms did not provide evidence for any widespread excitation of phrenic motoneurons by inspiratory interneurons. In addition, the cross-correlograms between interneurons did not provide evidence for a strong synchronizing input. Of the ten cross-correlograms between pairs of C5 inspiratory interneurons, only one had a peak centred about time zero, indicative of a common activation. A synchronizing input could result from either a single excitation source shared by both interneurons, such as synaptic input from collateral arborizations of a single medullary inspiratory neuron, or from excitation by different, but synchronized, sources for each interneuron, such as two synchronized medullary inspiratory neurons (e.g. Davies et al. 1985a). Altogether, these results suggest that such synchronizing inputs are weak or absent.

The results therefore indicate that the C5 inspiratory interneurons that we examined do not convey either central (medullary) inspiratory drive to phrenic motoneurons or intersegmental responses to stimulation of cervical and thoracic afferents.

Davies et al. (1985b) calculated that approximately 78% of the synaptic input to phrenic motoneurons is mediated via interneurons. If this is so, some of the cross-correlograms would be expected to have short-latency, narrow peaks indicating the presence of monosynaptic projections from interneurons to motoneurons. Despite the high counts and relatively low "noise" levels in many cross-correlograms, and our previous success in using this method to detect monosynaptic connections between medullary inspiratory neurons and the thoracic motoneurons in the cat (Duffin and Lipski 1987) and between medullary inspiratory neurons and phrenic motoneurons in the rat (Duffin and van Alphen 1995), we detected no such peaks. In addition, the estimated degree of interaction between interneurons and the ipsilateral and contralateral phrenic motoneurons that can have been missed in these cross-correlograms was low.

There could be other reasons why we did not detect connections from inspiratory interneurons to phrenic motoneurons. First, the population of inspiratory interneurons may have been sampled incorrectly. The recordings were made using tungsten microelectrodes positioned so as to record units with a high signal-to-noise ratio and well separated from phrenic motoneurons. If the interneurons that transmit respiratory drive are close to or intermingled with phrenic motoneurons, they will be missed. However, such interneurons have not yet been detected by us or others (e.g. Bellingham and Lipski 1990). The inspiratory interneurons recorded were not Renshaw cells, which discharge with a decrementing high-frequency burst of activity when the phrenic nerve is stimulated (Hilaire et al. 1983; Lipski et al. 1985); all the recorded neurons were so tested, and the one example resulting in a decrementing high-frequency burst of activity was excluded from analysis. The inspiratory interneurons from which we recorded may transmit central respiratory drive to phrenic motoneurons in other spinal segments, but in this case the inspiratory interneurons for phrenic motoneurons in the C5 segment must be located elsewhere and have not yet been discovered.

Second, some of the neurons may have been incorrectly classified as interneurons and may have been motoneurons, either phrenic motoneurons not antidromically activated, owing to damage during dissection, for example, or accessory motoneurons (Satomi et al. 1985).

Finally, the cross-correlation technique is thought to be less sensitive to slower, low-amplitude excitatory postsynaptic potentials (Aertsen and Gerstein 1985; Kirkwood 1979). If we consider, as a hypothesis, that these interneurons modulate phrenic motoneuron discharge via slow, low-amplitude, excitatory postsynaptic potentials, then cross-correlation will not detect such transmission. Should this be the case, it would necessitate a change in the current concept of transmission of respiratory drive by these interneurons.

In conclusion, although C5 segment inspiratory interneurons are active, discharging in phase with respiration, their role remains obscure. There is no evidence from this study that these neurons convey central respiratory drive to C5 phrenic motoneurons.

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