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AXONAL PROJECTIONS FROM THE ROSTRAL EXPIRATORY NEURONES OF THE BÖTZINGER COMPLEX TO MEDULLA AND SPINAL CORD IN THE CAT

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SUMMARY

1. Axonal projections of eighty-four rostral medullary expiratory neurones of the Bötzinger complex were tested using antidromic mapping techniques in anaesthetized cats.

2. A projection to the ventral respiratory neurones of the medulla (n.r.a.) was shown in eleven out of twelve tested neurones. Also a spinal projection to the C5–C6 cervical segments was evident in more than 72% of tested neurones; probably near 100% project to cervical cord. These axonal projections were found bilaterally in both brain stem and spinal cord.

3. The majority of Bötzinger complex expiratory neurones were seen to have two to four axonal collaterals to the ventro-lateral (v.l.) nucleus of the solitary tract (n.t.s.) and/or the n.r.a. and/or the spinal cord.

4. In eight out of twelve of the tested neurones, electrophysiological evidence of axonal arborization in more than one of n.r.a. inspiratory, n.r.a. expiratory or v.l. n.t.s. regions was obtained. Similar evidence for the terminal arborization was found for 26% of tested neurones in the phrenic motor nucleus.

5. The descending spinal expiratory axons of the Bötzinger complex neurones are located in the dorsal and medial parts of the lateral funiculus in C4 and C5 segments. Conduction velocity measurements indicate that these are large myelinated axons.

6. We propose that the Bötzinger complex expiratory neurones are a source of synaptic inhibition for n.r.a. inspiratory neurones and phrenic motoneurones.

INTRODUCTION

Interactions between different types of respiratory neurones in the medulla and spinal cord have been discussed in recent reviews (Cohen, 1979; Kalia, 1981; Wyman, 1977) and experimental papers (Merrill, 1974; Richter, Camerer, Meesman & Röhring, 1979). Until recently it seemed that all nuclei in the medulla with phasic, respiratory related neuronal activity had already been discovered. Anatomical data, however, revealed the existence of an afferent projection to the nucleus of the solitary tract (n.t.s.) from the most rostral contralateral and ipsilateral part of the nucleus ambiguus (Bystrzycka, 1980; Kalia, Feldman & Cohen, 1979). This pathway, as

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described by Merrill, Lipski, Kubin & Fedorko (1983), originates almost entirely from expiratory neurones located in the vicinity of the retrofacial nucleus (r.f.n.). The term 'Bötzinger complex' has been applied to respiratory neurones in this region, to distinguish them from (a) those neurones more strictly associated with the retrofacial nucleus which is generally slightly more lateral (Fig. 3*B*, see also Merrill *et al.* 1983), and from (b) respiratory modulated neurones of the rostral pole of the ambigual group, which are either laryngeal and pharyngeal motoneurones, or interneurones concerned with accessory respiratory movement (Merrill, 1970). The demonstration that the projection to n.t.s. from the retrofacial region (Kalia *et al.* 1979) is specifically from the Bötzinger expiratory neurones (Lipski & Merrill, 1980; Bianchi & Barrillot, 1982) and not those of either (a) or (b) confirms the utility of the label. We do not wish, however, to restrict the term only to neurones wth expiratory activity, although in our experience there are very few neurones with inspiratory patterns among the Bötzinger expiratory cells which project to n.t.s. (at least in the barbiturate-anaesthetized cat). Merrill *et al.* (1983) showed that the Bötzinger complex neurones are one source of the synaptic inhibition of the inspiratory neurones of the n.t.s. reported by Richter *et al.* (1979). The absence of any projection from expiratory neurones of the retroambigual nucleus (n.r.a.) to the ventro-lateral (v.l.) n.t.s. had been previously shown (Kalia, Sommer & Cohen, 1981; Merrill, 1974).

The question is: do these rostral medullary expiratory neurones, the Bötzinger complex, also inhibit other inspiratory neuronal populations in the medulla? Also there is the possibility that these inhibitory neurones could be responsible for the post-synaptic inhibition in phrenic motoneurones described by Berger (1979). The present experiments were performed in an attempt to find the necessary substrate for such synaptic actions: axonal projections from the Bötzinger complex expiratory neurones, using antidromic mapping techniques. Some of these results have been reported in an abstract (Fedorko, 1982).

METHODS

Experiments were performed on twenty cats (2·0–3·0 kg) anaesthetized with sodium pentobarbitone, 45 mg/kg I.P. Supplementary doses were injected I.V. (3–5 mg/kg . h). Tracheostomized animals were subjected to parietal craniotomy (see also Merrill *et al.* 1983), occipital craniotomy exposing the dorsal medulla, and cervical and thoracic laminectomy at C4, C5, C6 and Th1, Th2 segment levels respectively. Preparations were spontaneously breathing in most experiments. When paralysed (Flaxedil, 10 mg/kg . h I.V.), artificial ventilation was used; bilateral pneumothorax and C5 phrenic root dissection were performed. In spontaneously breathing animals a mercury strain gauge (Shapiro & Cohen, 1965) was used for monitoring respiratory phase.

Platinum-plated tungsten micro-electrodes (Digitimer Ltd.) were used to record neuronal activity and for microstimulation in the medulla and spinal cord. Two criteria were employed in selecting neurones for mapping. First, the recording electrode was placed in the retrofacial area previously identified by Kalia *et al.* (1979). Secondly, small adjustments in electrode position were then made until a population of neurones with characteristic late expiratory patterns, usually with fairly high peak firing rates, could be recorded. Neurones with these patterns, in this location, were previously shown to have a high connectivity with the contralateral n.t.s. (Lipski & Merrill, 1980; Merrill *et al.* 1983). Once the electrode was in such a suitable site all active neurones were then tested for projections; inspiratory neurones were rare in such groups, under the anaesthetic conditions used, and none are described here.

The antidromic mapping technique (Merrill, 1971; Jankowska & Roberts, 1972; Lipski, 1981) was used to determine axonal projections. Proper placement of the stimulating electrode in the

medullary respiratory nuclei (v.l. n.t.s. and n.r.a.) was achieved using anatomical coordinates with reference to the obex and the dorsal surface of the medulla (Berman, 1968) as well as by recording neuronal activity from the same electrode. A series of closely spaced (150–300 μm) penetrations were performed in each nucleus with alternate recording and stimulation. In the spinal cord the tracks of the stimulating electrode were 300–400 μm apart in the transverse direction. 3–50 μA and 3–100 μA stimulating current pulses (50–100 μs duration) were applied through the stimulating electrodes in the medulla and spinal cord respectively. Collision tests and determination of following frequency (Fuller & Schlag, 1976) (Fig. 1) were used routinely to verify the character of the responses to stimulation. Histological confirmation of the recording and stimulation sites was achieved by electrolytic lesions in the tested places at the end of the experiment. The antidromic mapping of the axonal projections of the tested neurones was conducted in the contra- and ipsilateral dorsal and ventral respiratory group and contra- and ipsilateral spinal cord at cervical (C4–C6) and thoracic (Th1–Th2) levels.

TABLE 1. Medullary projections from the Bötzinger complex

Stimulation place	Neurones tested	Antidromically activated	Percentage	Latency (ms)	Mean latency (ms)
N.t.s.	12	8	67		
Contralateral n.t.s.	12	7	58	0·3–1·6	1·0
Ipsilateral n.t.s.	4	2	50	0·6, 1·0	
N.r.a.	12	11	91		
Contralateral n.r.a.	9	6	67	0·2–2·3	1·3
Ipsilateral n.r.a.	10	7	70	0·5–6·0	1·7
Cervical spinal cord	64	46	72†	1·2–6·9*	2·8
Contralateral spinal cord	32	20	63		
Ipsilateral spinal cord	49	28	57		
Thoracic spinal cord	19	2	11	1·6, 3·2	

* Latencies measured at C5 spinal segment on both sides of the spinal cord.
† See Results.

RESULTS

Eighty-four expiratory neurones of the Bötzinger complex were tested. Twelve were tested by stimulation in the region of the respiratory medullary nuclei (contra- and ipsilateral n.t.s. and n.r.a.); seventy-two neurones were tested for projections to cervical and/or thoracic spinal cord (eleven of these in both cervical and thoracic cord).

Medullary projections from the Bötzinger complex

Out of twelve tested neurones, seven (63 %) could be activated from contralateral v.l. n.t.s. Nine neurones were tested additionally by stimulation in the region of contralateral n.r.a. and six (67 %) could be activated antidromically. Ten neurones from the same sample were tested by stimulation in the ipsilateral n.r.a. and seven (70 %) were antidromically activated. Two units were activated antidromically from the ipsilateral n.t.s., but due to technical reasons only four neurones were fully tested in this region. The number of tested neurones is shown in Table 1.

Seven neurones from the same sample were also quickly tested by stimulation in the C3 spinal segment and four of them had an axon in this region (because of the cursory nature of the spinal tests on these they are not included in the cervical sample in the Table).

In eight units it was possible to show the existence of more than one axonal branch (Fig. 1): five units could be activated from two, two units could be activated from three and one unit could be activated from four different places in the medulla or spinal cord.

In seven tested neurones more than one antidromic latency could be observed during stimulation among n.r.a. respiratory neurones, by using different strengths

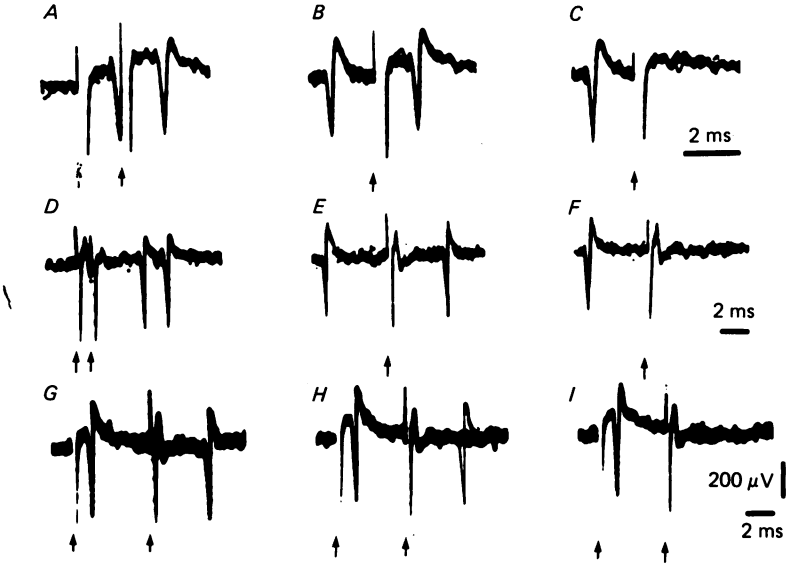

Fig. 1. Antidromically evoked action potentials in a Bötzinger complex expiratory neurone. *A*, stimulation in the contralateral v.l. n.t.s. with paired stimuli (arrows) at the maximal frequency for activation by both stimuli. *B* and *C*, the collision between the spontaneous and the antidromically evoked action potentials when delay of the stimulus was shortened by 0·2 ms. *D*, *E* and *F*, same as in *A*, *B* and *C*, except that C3 cervical spinal cord was stimulated. *G*, *H* and *I*, the collision between two antidromically evoked action potentials (first stimulus of the pair in the v.l. n.t.s., second in the spinal cord). Each trace consists of five superimposed sweeps. Vertical calibration bars 200 μV, horizontal bars 2 ms.

of stimuli or changing the position of the electrode within the same track. This evidence for axonal arborization could be observed in inspiratory as well as expiratory parts of n.r.a. The same kind of variations in latency were recorded in the region of the ispilateral and contralateral v.l. n.t.s. These changes in antidromic latency were usually greater (ranging from 0·2 to 4·0 ms) than the shift in the antidromic latency occurring during changes in the level of excitability of the Bötzinger expiratory neurones. The latter variations in the latency depend on initial segment–somodentritic (i.s.–s.d.) delays in soma invasion (Gustafsson & Lipski, 1980). In Bötzinger complex neurones the i.s.–s.d. delay variation rarely exceeds 250 μs between expiratory and inspiratory phases.

Spinal axonal projection from Bötzinger complex neurones

Projection to the cervical spinal cord. Seventy-two expiratory neurones of the Bötzinger complex were tested by stimulation of the spinal cord. Forty-six (72 %) of

these neurones could be antidromically activated from the C3–5 spinal segment: twenty-eight (57 %) out of forty-nine tested on the ipsilateral side of the spinal cord had spinal projection while twenty (63 %) out of thirty-two neurones tested on the contralateral side, had spinal projection. When penetrations were made across the entire width of the spinal cord at C4–5 (sixteen neurones), axon collaterals could be shown on both sides of the spinal cord for two neurones.

The following analysis suggests that close to 100 % of all Bötzinger axons must project to the cervical cord:

$$63\,\%\ (\text{Table 1}) = b\ (\text{bilateral}) + c\ (\text{contralateral only})$$

$$57\,\%\ (\text{Table 1}) = b + i\ (\text{ipsilateral only})$$

$$P\ (\text{total projection}) = b + c + i.$$

From our bilaterally tested sample, two could be shown to project bilaterally (i.e. $b = 13\,\%$). Thus $c = 50\,\%$, $i = 44\,\%$ and $P = 107\,\%$. Obviously P cannot be greater than 100 %, and we suspect that 13 % underestimates the bilateral projection. That the 72 % shown in Table 1 is underestimated is further confirmed by the fact that fifteen out of sixteen bilaterally tested cells projected, though the sample is small. Our small sample size and restricted testing allows only approximate estimates of extent of these projections.

Twenty-one expiratory neurones projecting to the C5 spinal segment were further tested with stimulation in the phrenic nucleus. (The phrenic nucleus was located by recording the antidromic field potential through the stimulating electrode when the phrenic C5 root was stimulated.) More than one antidromic latency could be shown in six (29 %) tested neurones.

Projection to the upper thoracic spinal cord. Axonal projection from the Bötzinger complex to the thoracic spinal cord was investigated on a sample of nineteen neurones. Only two (10 %) could be antidromically activated from the Th1–2 spinal level. In both these cases the units were activated by stimulation of the ipsilateral spinal cord. All of the tested neurones could be synaptically activated from that level by stimulation within the ipsilateral lateral funiculus when the stimulating current was greater than 100 μA. The latency of this synaptic response was similar in all neurones and varied from 4 to 5 ms.

Conduction velocity. Measurements were made to assess the conduction velocities of the tested axons. Short latencies were encountered for medullary stimulation (Table 1); only the shortest response latency was considered to be the result of activation of the main axonal branch. Responses evoked by stimulation of smaller branches of the axonal arbors in the region of the n.t.s. had longer latencies and varied from 0·7 to 4·5 ms. For axons projecting to n.r.a., the shortest latencies varied from 0·2 to 2·3 ms (mean 1·3 ms) while a delay of 6·0 ms in the antidromic response could be observed when one of the arbors in the ipsilateral n.r.a. was stimulated. Because the antidromic latency is dominated by the i.s.–s.d. invasion time (some 250 μs) for medullary stimulation, accurate conduction velocity estimates cannot be made for medullary branches.

Latency measurements were performed for cervical and thoracic stimulation, where latencies of the antidromic responses are considerably longer (Fig. 2). At C5 spinal level stimulation, latency of the antidromic evoked potentials varied from 1·2 to

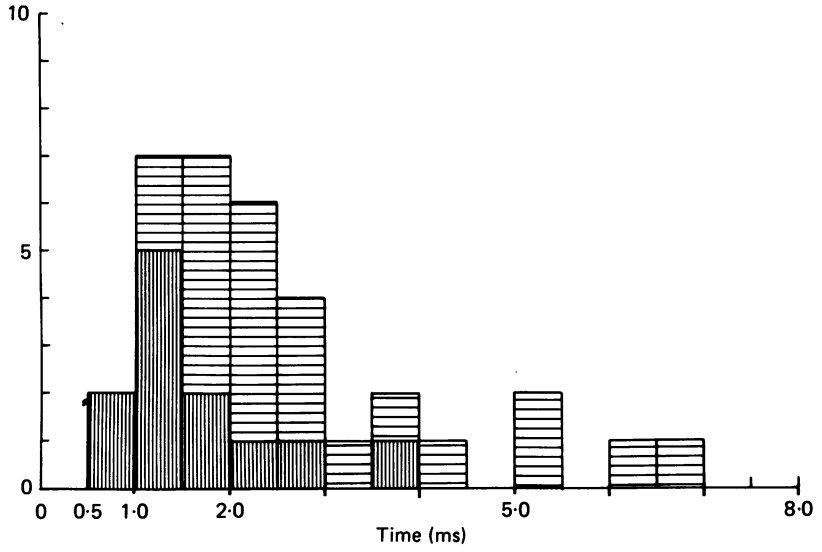

Fig. 2. Latencies of the antidromic evoked potentials when v.l. n.t.s. was stimulated (vertically hatched area) and when C5 spinal segment was stimulated (horizontal hatching).

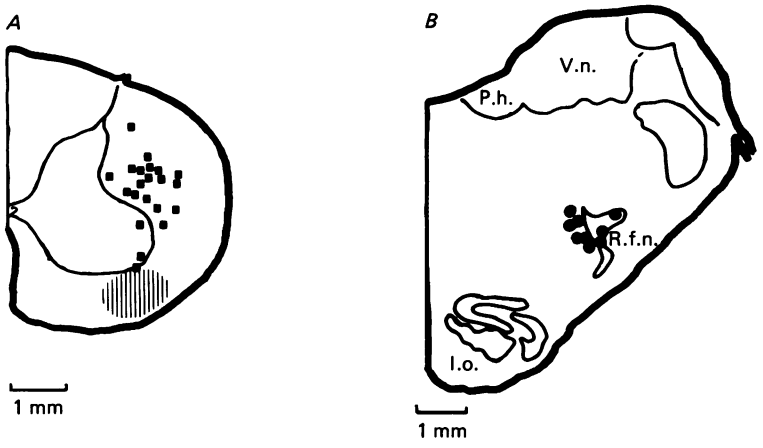

Fig. 3. *A*, localization of the descending axons of the Bötzinger complex expiratory neurones (filled squares) and n.r.a. expiratory axons (hatched area, Merrill, 1971) at C4–5 segment. *B*, localization of each of the marked Bötzinger complex recording sites. Plane of section, 4·0 mm rostral to the obex (Berman, 1968); r.f.n., retrofacial nucleus; i.o., inferior olive; v.n., vestibular nucleus; p.h., parahypoglossal nucleus. Calibration bar, 1 mm.

6·9 ms (mean 2·8 ms). Calculated conduction velocities varied from 8·7 to 48 m/s. No corrections for stimulus utilization time or soma invasion times were made in calculations.

Histology. Locations of the recording sites within the Bötzinger complex were determined by electrolytic lesions at the recording site, made at the end of nine experiments. Results confirmed that the recorded units were in the same area as shown

in previous papers (Lipski & Merrill, 1980; Merrill *et al.* 1983) in the vicinity of the r.f.n. (Fig. 3*B*).

Location of the descending spinal axons was established by electrolytic lesions made at the lowest threshold foci. All axons were found in the contra- and ipsilateral lateral funiculi of the cervical C4–5 segment (Fig. 3*B*). Axons were scattered and did not form the well circumscribed bundle characteristic of the axons of n.r.a. expiratory cells at the C3 level (n.r.a. axons begin to disperse by C5, but not to the extent of those in Fig. 3, Merrill, 1971, 1974). The spinal axons from the Bötzinger complex expiratory cells tend to be found much more dorsal than n.r.a. axons.

DISCUSSION

The expiratory neurones of the Bötzinger complex have been shown to project to the ipsi- and contralateral v.l. n.t.s. and n.r.a. respiratory regions. This extensive projection may be responsible for post-synaptic inhibition of n.r.a. as well as n.t.s. inspiratory neurones (Richter & Heyde, 1974; Richter *et al.* 1979). The inhibitory post-synaptic potentials in these neurones have a late expiratory ramp pattern similar to the pattern of the activity of the Bötzinger complex expiratory neurones (Lipski & Merrill, 1980). Indeed, Bötzinger neurones have recently been shown to monosynaptically inhibit the inspiratory neurones in the contralateral v.l. n.t.s. (Merrill *et al.* 1983). The present data indicate that branching of some expiratory Bötzinger complex axons can be found in the contralateral v.l. n.t.s., in the n.r.a. on both sides of the medulla and also in the ipsilateral v.l. n.t.s. It is likely that these projections, shown to be inhibitory in the contralateral v.l. n.t.s., are also inhibitory for inspiratory neurones in other medullary nuclei.

Recent data from experiments on monkeys, cats and rabbits (Karczewski & Gromysz, 1981, 1982; Kubin, 1981; St John, 1983, personal communication) describe desynchronization of respiratory rhythm generation between the two halves of the medulla after mid-line saggital sections of the medulla, rostral and caudal to the obex. Thus, the bilateral projection from the Bötzinger complex expiratory neurones to all inspiratory populations in the medulla may play an important role not only in maintaining the inactive expiratory phase in these neurones but also in the synchronization of rhythm generation in both sides of the medulla.

Gill & Kuno (1963) did not find expiratory post-synaptic inhibition in phrenic motoneurones. Zieliński & Gebber (1975), however, using indirect methods described a supraspinal inhibitory input to the phrenic motoneurones. This inhibition had a late expiratory ramp pattern. These observations were confirmed by intracellular recordings (Berger, 1979) which showed prominent late expiratory inhibitory post-synaptic potentials (i.p.s.p.s) in phrenic motoneurones. The i.p.s.p.s were readily reversed by hyperpolarization. The present results provide strong evidence that Bötzinger complex expiratory neurones form a long descending inhibitory pathway to phrenic motoneurones. First, we have shown the presence of the bilateral expiratory descending pathway to the cervical spinal segments from neurones already known to be inhibitory for medullary inspiratory neurones. Secondly, there is a striking similarity between the firing patterns of these neurones and the pattern of i.p.s.p.s in phrenic motoneurones. Thirdly, axonal branching within the phrenic

nucleus of descending Bötzinger complex axons has been shown. Branching in the phrenic nucleus could not be shown for caudal n.r.a. expiratory neurones (Merrill, 1974).

The surprisingly low porportion of axons with arbors in the phrenic nucleus (26 %), compared with the very large proportion of Bötzinger complex axons projecting to the C5 spinal segment, does not exclude the possibility that most of them make synaptic contact with dendrites of phrenic motoneurones since the phrenic dendritic trees extend beyond the phrenic motor nucleus (Cameron, Averill & Berger, 1983). Our own recent preliminary data (Merrill, 1982) provide additional support for monosynaptic inhibition of phrenic motoneurones by Bötzinger complex expiratory neurones.

The present results indicate that a larger proportion of Bötzinger complex expiratory neurones project bilaterally to n.r.a., v.l. n.t.s. respiratory nuclei and the spinal cord than was recently reported by Bianchi & Barillot (1982). Differences in the results can be partially explained by differences in the antidromic mapping techniques used: gross bipolar stimulation of the spinal cord can fail to reveal axons which are readily demonstrated with monopolar microstimulation (Lipski, 1981), particularly in view of the scattering of Bötzinger expiratory spinal axons within the lateral funiculus. The other reasons for these differences may be slightly different sampling locations in the cited study.

Axonal projections to thoracic levels could not be shown for most of the tested Bötzinger complex neurones, in contrast to the readily obtained cervical antidromic activation. These observations suggest that most descending axons from the Bötzinger complex end in the cervical segments at the level of the phrenic nucleus.

Expiratory neurones of the caudal n.r.a. are considered to be the excitatory interneurones for contralateral intercostal expiratory motoneurones (Kirkwood & Sears, 1973; though see Lipski & Merrill, 1983). Extensive antidromic mapping experiments (Merrill, 1974, 1979) failed to show any axonal branching in the medulla and the cervical spinal cord for these neurones, and those results were confirmed recently by horseradish peroxidase anatomical techniques (Kalia *et al.* 1981). The presence of Bötzinger axonal arbors not only in the inspiratory nuclei of the medulla, but also in the purely expiratory caudal n.r.a., suggest that they may have a role in shaping the firing patterns of caudal n.r.a. expiratory neurones. Alternatively, both neuronal populations may receive a common synaptic input which underlies their strikingly similar firing pattern, but then the function of the expiratory n.r.a. projection from Bötzinger complex cells requires explanation.

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