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MESENCEPHALIC STIMULATION ELICITS INHIBITION OF PHRENIC NERVE ACTIVITY IN CAT

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SUMMARY

1. Previous work from this laboratory has indicated that the mesencephalon is the anatomical substrate for a mechanism capable of inhibiting central respiratory drive in glomectomized cats for periods of up to 1 h or more following brief exposure to systemic hypoxia; phrenic nerve activity was used as an index of central respiratory drive.

2. The present study was undertaken to further localize the region responsible for the observed post-hypoxic inhibition of respiratory drive. We studied the phrenic nerve response to stimulations of the mesencephalon in anaesthetized, paralysed peripherally chemo-denervated cats with end-expired P_{CO_2} and body temperature servo-controlled.

3. Stimulations of two types were employed. Electrical stimulation allowed rapid determination of sites from which phrenic inhibition could be elicited. Microinjections of excitatory amino acids were used subsequently in order to confine excitation to neuronal cell bodies and not axons of passage.

4. Stimulation of discrete regions of the ventromedial aspect of the mesencephalon in the vicinity of the red nucleus produced substantial inhibition of phrenic activity which lasted up to 45 min. Stimulation of other areas of the mesencephalon either produced no phrenic inhibition or resulted in a slight stimulation of phrenic activity.

5. The results are discussed in the context of the central respiratory response to hypoxia.

INTRODUCTION

This laboratory has reported a long-lasting inhibition of central respiratory drive in glomectomized cats following brief (10 min) exposure to hypoxia (Millhorn, Eldridge, Kiley & Waldrop, 1984). This inhibition lasts up to 1 h or more following cessation of the hypoxic insult. Further investigation revealed that the mesencephalon is required, but that structures further rostral are not necessary, for expression of this long-lasting post-hypoxic inhibition (Gallman & Millhorn, 1988). Other investigators have similarly concluded that a suprapontine mechanism is involved in depression of respiration, particularly in fetal and neonatal animals (Dawes, Gardner, Johnston & Walker, 1983; Martin-Body & Johnston, 1988; Martin-Body, 1988).

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As early as 1936, Kabat made an extensive exploration of the forebrain and mesencephalon of cat, using electrical stimulation. He reported a decrease in ventilation in response to a number of stimulations, many of them located in the vicinity of the medial part of the mesencephalic central tegmentum. Baxter and Olszewski (1955) also reported decreases in respiratory activity in cat in response to electrical stimulation of brain stem. While most of their stimulation sites were pontine, a few involved the caudal mesencephalon, including one site on the dorsolateral border of the red nucleus. Electrical stimulation of the ventromedial mesencephalon, in particular the mesencephalic tegmentum, has also been shown to cause respiratory inhibition in rabbit (Evans & Pepler, 1974). In contrast, both electrical and excitatory amino acid (EAA) stimulation of sites in the dorsal aspect of the mesencephalon have been reported to stimulate respiratory activity in rat Bandler, Depaulis & Vergnes, 1985; Keay, Redgrave & Dean, 1988). These observations, coupled with observations made during our previously reported ablation studies (Gallman & Millhorn, 1988), led us to concentrate our search for the substrates of hypoxia-induced respiratory inhibition in the ventromedial mesencephalon.

The present study was undertaken to further localize areas in the mesencephalon which could contribute to the long-lasting inhibition of respiration following hypoxia in adult cat. In particular, we wished to determine whether stimulation of cell bodies in any region of the mesencephalon could produce inhibition of phrenic nerve activity. For this purpose, we have made use of the excitatory amino acids L-glutamate and L-aspartate which excite cell bodies but not axons of passage (Zieglgänsberger & Puil, 1973; Goodchild, Dampney & Bandler, 1982).

We here report that it is, indeed, possible to elicit inhibition of phrenic nerve activity in response to EAA stimulation of cell bodies in the ventromedial mesencephalon. This response appears to be evocable from discrete regions in the vicinity of the red nucleus. While most injections elicited responses of small magnitude and duration, on occasion the response to a single injection was long-lasting and resembled post-hypoxic respiratory depression in both magnitude and duration.

METHODS

Studies were performed in ten cats of both sexes weighing 2·7–5·0 kg. Deep surgical anaesthesia was induced with ether followed by chloralose (40 mg kg^{-1}) and urethane (250 mg kg^{-1}) administered through a catheter introduced into a femoral vein. A second catheter, introduced into a femoral artery, was used to monitor arterial blood pressure. Thus, both blood pressure and pupillary response were available to assess the level of anaesthesia. All animals were maintained continuously under deep surgical anaesthesia, as determined by a lack of pupillary reflex and an absence of cardiovascular reflex in response to painful stimuli.

Expired air was sampled continuously through a cannula inserted into the trachea and end-tidal P_{CO_2} analysed by an infra-red analyser (Beckman LB-2). The cats were paralysed with gallamine triethiodide (3 mg kg^{-1} I.V. initially, followed by a continuous infusion at the rate of 3 mg kg^{-1} h^{-1}) and artificially ventilated. Ventilator pump rate was servo-controlled to maintain end-expired P_{CO_2} at any desired level $\pm 0{\cdot}5$ Torr. A rectal thermistor was used to monitor body temperature. The thermistor was interfaced to a servo-control unit and heating pad which maintained body temperature at $37{\cdot}5 \pm 0{\cdot}5$ °C. The carotid sinus nerves were identified visually and cut, as were the vago-sympathetic trunks.

A phrenic nerve root (C5) was isolated, cut, desheathed and the proximal end wrapped around a pair of platinum recording electrodes. The electrodes were contained within a Teflon platform, the only external attachment of which was a flexible cable leading to a preamplifier (Grass). With this flexible arrangement, it was possible to maintain a constant electrical coupling between the nerve and the electrodes for extended periods of time.

Phrenic nerve activity was amplified, full-wave rectified, and integrated over intervals of 100 ms using a sample-and-hold integrator (Gould). Peak phrenic nerve activity, the neural equivalent of tidal volume (Eldridge, 1975), was determined for each breath. Minute phrenic activity was calculated by multiplying peak phrenic activity by phrenic 'respiratory' rate.

Each animal was placed in a stereotaxic head holder (Kopf) and a section of the skull was removed extending from the tentorium 10 mm rostral and 5 mm to each side of the mid-line. One- or three-barrelled micropipettes were positioned stereotaxically and prepared for pressure pulse injection (General Valve Picospritzer II). The one-barrelled micropipettes were filled with 0·5 M-L-glutamate. The three-barrelled micropipettes all contained vehicle plus Fast Green dye in one barrel. The second barrel contained 0·5 M-L-aspartate. The third barrel contained either 1·0 M-L-aspartate or 0·5 M-L-glutamate. The vehicle in each case was either lactated Ringer solution or phosphate-buffered saline, with pH adjusted to 7·4. In addition to marking locations, injections of vehicle containing Fast Green dye served as control injections. Injection volumes, measured directly using a monocular microscope equipped with a reticule in the eyepiece, ranged from a minimum of 40 nl to a maximum of 470 nl (one injection). Sixty-six per cent of all injections were 100 nl or less.

In some animals, electrical stimuli (1·0–10·0 V, 50 or 100 Hz, 0·5 ms pulse duration) restricted to the left half of the mesencephalon, were made in order to determine optimal locations for eliciting respiratory inhibition. Amplitude and frequency of these electrical stimuli were chosen so as to maximize change in phrenic nerve output while minimizing concomitant change in arterial blood pressure. Amino acid injections in these animals were restricted to the right half of the mesencephalon. This procedure increased our chances of activating the inhibitory response with the first or second EAA injection. Micropipette or microelectrode tip placement was determined using stereotaxic co-ordinates referenced to interaural (IA) zero. Tip location relative to mesencephalic nuclei and anatomical structures was estimated prior to injections using the atlas of Berman (1968).

All parameters (integrated phrenic nerve activity, airway P_{CO_2}, arterial blood pressure and a marker indicating times of injections) were recorded on a strip chart recorder (Gould) and were simultaneously analysed by computer. Thus, both breath-by-breath analysis of phrenic activity and averaged values were available with the corresponding values for end-tidal P_{CO_2} and mean arterial pressure. Phrenic nerve activity was normalized by assigning a value of 70 units to the phrenic activity measured at a P_{CO_2} 20 Torr above apnoeic threshold and a value of 0 units to baseline activity at phrenic apnoea (Eldridge, Gill-Kumar & Millhorn, 1981). In addition, raw phrenic nerve activity, arterial blood pressure, tracheal P_{CO_2} and the injection marker were recorded on cassette tape for subsequent additional analysis.

Access to immediate computer analysis made it possible to determine quantitatively the stability of the animal prior to each injection. Once stability had been assured, 1–2 min of data were collected as a control. The pressure injection of EAA or vehicle was then made, generally over a period of 5–20 s. The response was recorded for the first 3–5 min following the injection. If, at the end of 5 min, phrenic activity had not returned to the control level, 1 min samples of data were collected at 10 min post-injection and every 5 min thereafter until phrenic activity returned to control. All responses following injections are reported here as change from control, expressed in normalized units.

In each animal, one or more tip locations were marked by injection of Fast Green dye in vehicle, the volume being matched to that of an EAA injection so that the effects of volume injection could be observed and recorded. In cases where prior electrical stimulation was employed, sites were marked by electrocoagulation.

Upon termination of the experiment, most animals were perfused transcardially with 4% paraformaldehyde and their brains removed. The mesencephalic region of each brain was sectioned on a microtome in a cryostat into 40 μm thick sections, mounted onto microscope slides and stained with Cresyl Violet. The position of the stereotaxically located injection sites was confirmed by locating the micropipette tracts, the dye injection sites, or the sites of electrocoagulation.

RESULTS

Electrical stimulations were used to locate regions of the mesencephalon from which it was possible to elicit inhibition of phrenic nerve activity. We found that many sites tested elicited various forms of stimulation of phrenic activity rather than inhibition. In general, these were the more dorsal sites. However, even within the ventral mesencephalon, many electrical stimulations yielded some form of facilitation of phrenic activity. Figure 1 illustrates the change in activity of the phrenic nerve when similar electrical stimulations were given at varying depths within one electrode track. As was often noted, stimulation at a more dorsal location (Fig. 1*A*) produced an increase in phrenic activity whereas stimulation 1 (Fig. 1*B*) or 2 (Fig. 1*C*) mm more ventral caused inhibition of phrenic activity.

Once sites had been located that could mediate phrenic inhibition, the contralateral side was then searched using EAA injections. For instance, an electrical stimulation given near the dorsomedial border of the red nucleus completely stopped phrenic nerve activity (Fig. 2*A*). An EAA injection at a similar location on the contralateral side evoked a prolonged decrease in phrenic nerve output (Fig. 2*B*).

This protocol, involving electrical stimulations followed by contralateral EAA injections, was employed in the initial experiments. A representative transverse section through the mesencephalon of one such brain is shown in Fig. 3. Damage from six microelectrode tracks is indicated on the left side of the section. On the right side, three micropipette tracks were evident. A Fast Green injection was given at 2·5 mm rostral, 1·5 mm lateral and 6 mm dorsal to IA zero, according to stereotaxic co-ordinates at the time of injection. The vertical co-ordinate of this location, approximated from the atlas of Berman, is closer to 7 mm dorsal to IA zero. This illustrates a common finding, namely that the actual site of the microelectrode or micropipette tip was often as much as 1 mm more dorsal than anticipated. Thus it is important that most injection sites (85%) were later located histologically. In the few cases where the location of the tip was not determined histologically, the anatomical location was considered to be the line between the stereotaxic vertical co-ordinate and 1 mm dorsal to that point. This correction was used in summarizing locations tested (e.g. Fig. 8). However, in any case where proximity to a known anatomical landmark is inferred (e.g. Fig. 6), injection sites were verified histologically.

A total of sixty-five EAA injection sites were studied. A response was classified as inhibition if the average phrenic activity for the first 5 min following the injection was 5 or more units below control. When an injection elicited an inhibitory response, the response was generally evident within a few seconds following the end of the injection and remained 5 or more units below control for at least 5 min after the injection. Figure 4*A* presents the raw data for one such injection. Phrenic activity decreased immediately following this injection and remained depressed for more than 10 min. By 15 min, phrenic activity had returned nearly to the control level. In this animal, a second injection at the same location, 20 min after the first injection, elicited a very similar inhibition (Fig. 4*B*). The complete response to each injection is presented graphically in Fig. 4*C*. In each case, maximal inhibition was reached between 5 and 10 min following the injection and phrenic activity then returned

towards the control level. As illustrated in Fig. 4*C*, the time courses of the initial inhibition following these two injections were very similar. In six of ten such instances, where a site found to produce inhibition was retested after 15 or more minutes, the second injection also produced an inhibitory response.

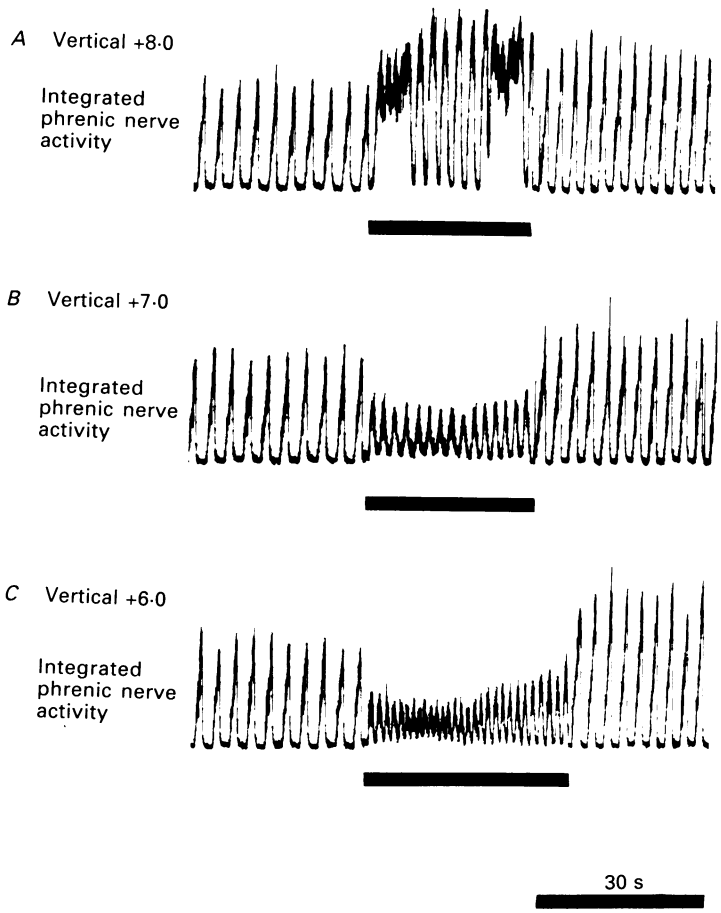

Fig. 1. Representative phrenic nerve responses to electrical stimulations given in ventromedial mesencephalon. Location was 3 mm rostral and 2 mm lateral to IA zero, depths as indicated. Stimulations, indicated by bars, were 4·0 V, 100 Hz, 0·5 ms pulse duration. Stimulation in *A* was changed briefly to 3·0, 3·5 and 5·0 V.

The inhibitory responses seen were not a result of pressure or volume of injection. In four animals, after inhibition had been demonstrated at a site, an injection of vehicle in the same volume was given at the same location. One such site (Fig. 5) produced an average decrease in minute phrenic activity of greater than 10 normalized units in response to an injection of 40 nl of 0·5 M-L-aspartate. An injection of 40 nl of vehicle plus Fast Green was given in the same location 20 min later. This control injection, as well as all other control injections, failed to produce a decrease in minute phrenic activity.

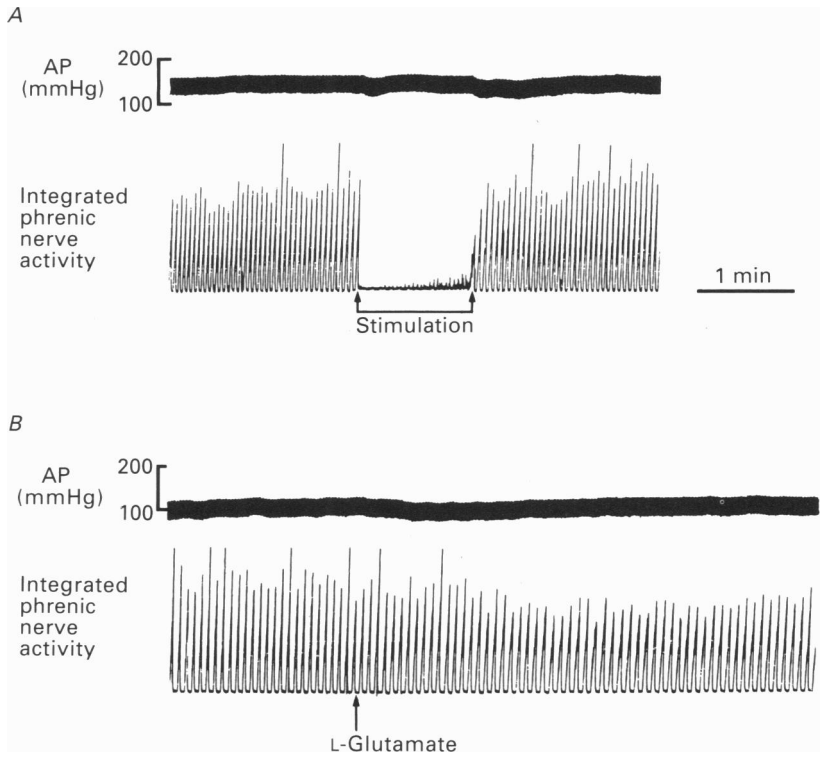

Fig. 2. Example of the use of electrical stimulation to select location of subsequent EAA injection. *A*, electrical stimulation at 4·0 mm rostral, 2·0 mm left lateral, and 7 mm above IA zero caused complete inhibition of phrenic nerve activity. Stimulation parameters: 6 V, 100 Hz, 0·5 ms pulse duration, stimulus duration 70 s. *B*, a subsequent EAA injection (120 nl 0·5 M-L-glutamate) at 4·0 mm rostral, 2·0 mm right lateral, 8 mm above IA zero also elicited phrenic inhibition. AP, arterial blood pressure.

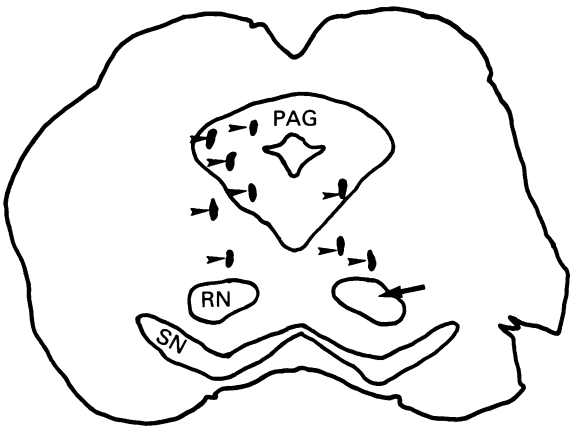

Fig. 3. Reconstruction of a representative transverse section through mesencephalon of cat. On the left, damage from six microelectrode tracks was visible; on the right side, evidence of three micropipette tracks was seen (Filled arrow-heads). A Fast Green injection (filled arrow) was given to mark the site of an EAA injection. (RN, red nucleus; PAG, periaqueductal grey; SN, substantia nigra)

Inhibition of phrenic activity, when elicited, was a site-specific phenomenon. Often, a location which could be stimulated to produce marked phrenic inhibition was within 1 mm of a site at which stimulation produced little or no change in phrenic activity. Multiple injections were often made in one track with one or more

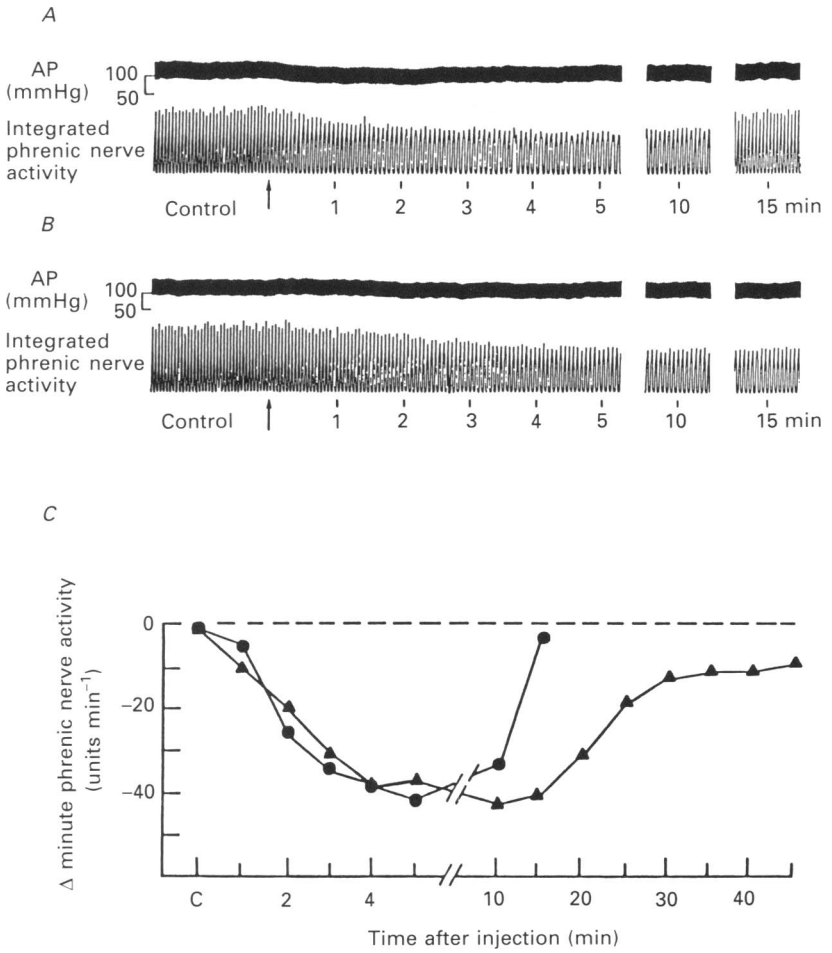

Fig. 4. Time course and repeatability of inhibition of phrenic nerve activity in response to activation of cell bodies located in the ventromedial mesencephalon. *A* and *B*, 120 nl of 1·0 M-L-aspartate. Each injection ended at arrow. Integrated phrenic nerve activity and arterial blood pressure (AP) are presented for the minute prior to injection, for the 5 min following the injection, and for 1 min at 10 and at 15 min following injection. Elapsed time between injections was 20 min. *C*, plot of minute phrenic nerve activity showing the time course of recovery from inhibition for injection in *A* (●) and injection in *B* (▲). C, control.

millimetres separating individual injection sites. We found that the likelihood of eliciting inhibition was greater in the ventral third of the mesencephalon, that is, at or below 8 mm above IA zero. Figure 6 illustrates the different responses elicited by

L-glutamate injection at varying depths within one micropipette track in one animal. Following a 160 nl injection given 8 mm above IA zero, no change in minute phrenic nerve activity was noted. However, at 7 mm and again at 6 mm above IA zero, a marked decrease in phrenic activity was obtained following injections of 80

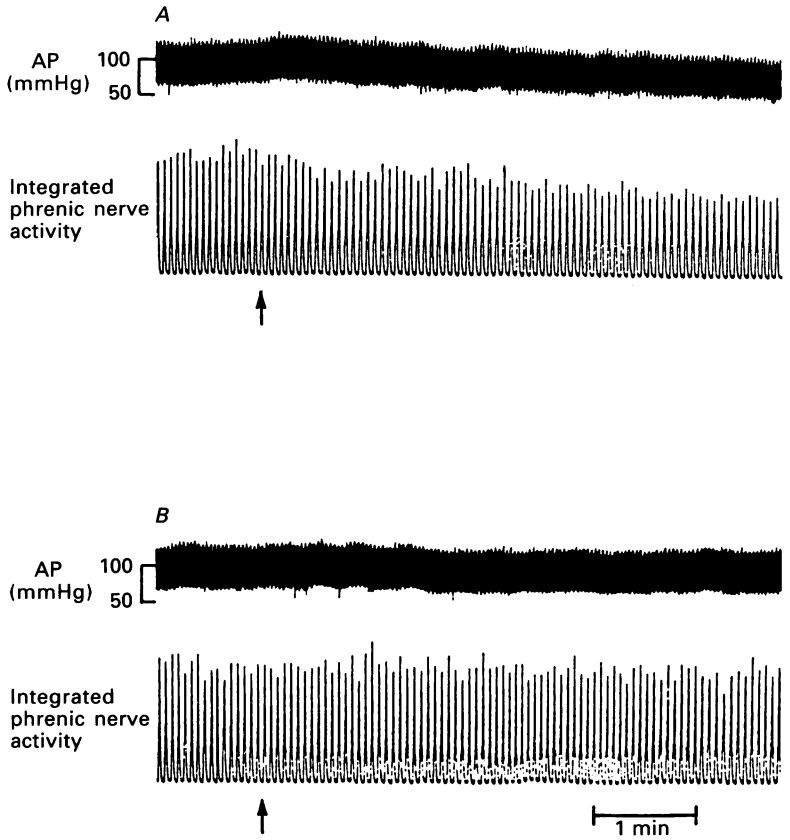

Fig. 5. Comparison of injection of excitatory amino acid and injection of vehicle. *A*, 40 nl 0·5 M-L-aspartate delivered 2·0 mm rostral, 2·5 mm lateral and 8 mm above IA zero evoked a clear decrease in phrenic nerve output. *B*, 40 nl vehicle delivered into the same location caused no change in phrenic nerve activity. Time elapsed between injections was 20 min.

and 120 nl, respectively. The degree of responsiveness to EAA injection also varied with the rostrocaudal location of the injection site. The three injections in Fig. 7 were all delivered at the same depth, roughly 7 mm above IA zero. At 3·5 mm rostral to IA zero, a small decrease in phrenic activity was seen in response to EAA injection (Fig. 7*A*). A larger decrease was seen when the injection site was 0·5 mm more caudal (Fig. 7*B*). The largest and the most rapid decrease was elicited 2·5 mm rostral to IA zero (Fig. 7*C*). This site was marked with Fast Green and is shown in Fig. 3.

All sixty-five injection sites were located within the box in Fig. 8. Roughly two-thirds of all injection site were located in the shaded region. In this shaded region,

35% of injections resulted in inhibition. Dorsal to this region, 8% of injections elicited inhibition, while the small area ventral to the shaded region produced inhibition following 15% of injections. The rostrocaudal extent of sites tested was from 1·0 to 4·5 mm rostral to IA zero. Roughly two-thirds of all sites tested were

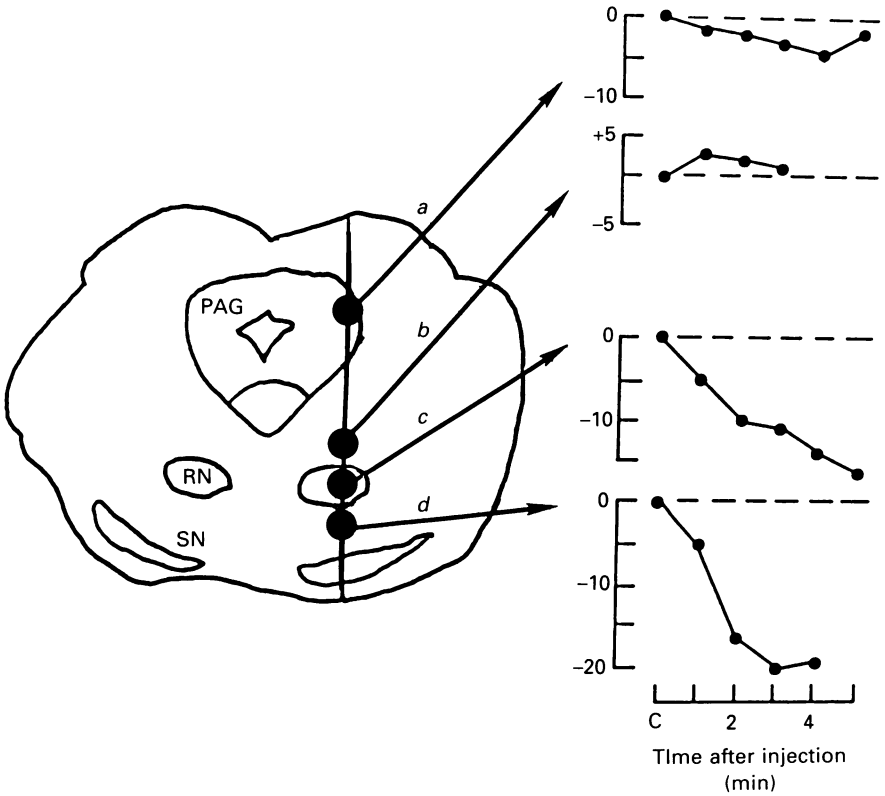

Fig. 6. Responses of one cat to a series of injections given in a single micropipette track. All injections were of 0·5 M-L-glutamate. Responses measured as change in normalized minute phrenic nerve activity. *a*, injection, total volume 120 nl, 12 mm above IA zero, evoked little change in phrenic output. *b*, injection, total volume 160 nl, 8 mm above IA zero, did not cause inhibition of phrenic activity. *c*, at 7 mm above IA zero, an 80 nl injection was sufficient to evoke a decrease in phrenic activity of more than 15 units. *d*, injection, total volume 120 nl, 6 mm above IA zero, caused a decrease in phrenic output of more than 20 units.

located between 2·0 and 3·0 mm rostral to IA zero. Of these, 24% elicited inhibition. Caudal to this region, two of five injections produced inhibition. Rostral to this region, 22% of injections resulted in inhibition.

We found that repeat injections in a given site gave unpredictable results. Results were more likely to be reproducible at a given injection site if a period of greater than 15 min elapsed between injections. Other investigators have made similar observations (Holtman, Anastasi, Norman & Dretchen, 1986). For this reason, we

consider separately the repeat injections where no injection had been delivered to that site within the previous 15 min and those which occurred less than 15 min after a prior injection at the same site. There were twelve repeat injections meeting the first criterion and twenty meeting the second. Thus, a total of ninety-seven EAA injections were studied.

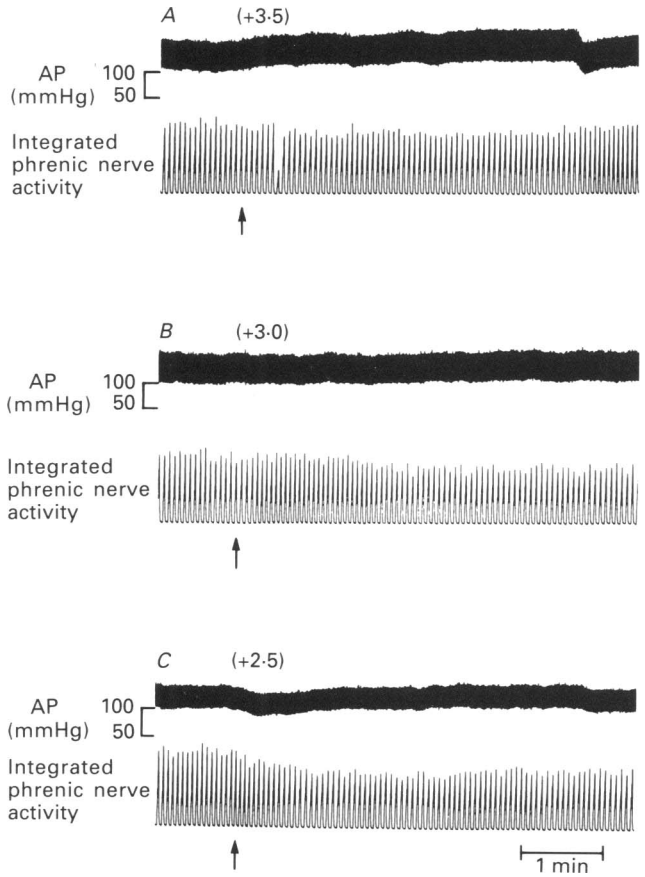

Fig. 7. Responses of one cat to a series of 0·5 M-L-glutamate injections all given at the same vertical depth. The location of injection *C* is indicated by the Fast Green injection in Fig. 3. *A*, at 3·5 mm rostral to IA zero, a 140 nl injection produced a small decrease in phrenic activity. *B*, at 3·0 mm rostral to IA zero, an 80 nl injection produced a greater decrease in phrenic activity. *C*, at 2·5 mm rostral to IA zero, an 80 nl injection evoked an even greater decrease in phrenic activity. At least 20 min elapsed between injections.

Of the twelve repeat injection sites with more than 15 min between injections, six elicited inhibition both times (e.g. Fig. 4), two produced no change in phrenic activity with either injection, and three which elicited inhibition with the first injection showed no response to the second. One site which caused inhibition after the first injection elicited stimulation the second time. None of the twenty injections

given within 15 min of a previous injection elicited inhibition, although ten of these injections followed injections which produced inhibition.

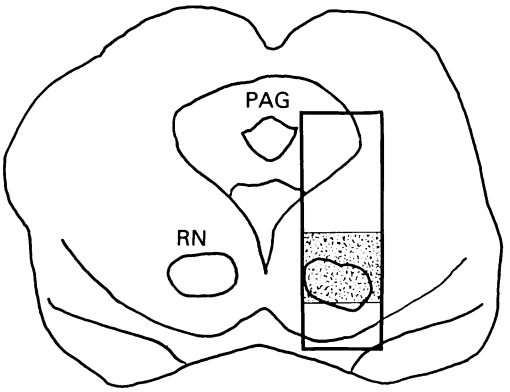

Fig. 8. Representative transverse section through mesencephalon. All seventy-seven injections, incorporating sixty-five injection sites, were located within the box. 68% of all injections fell within the shaded area. Within the shaded area, 35% of injections produced inhibition. Dorsal to this area, 8% of injections produced inhibition. Ventral to this area, 15% of injections produced inhibition. Overall, 23% of sites and 27% of injections produced inhibition.

DISCUSSION

The primary finding of this study is that stimulation of cell bodies in the ventromedial mesencephalon can elicit inhibition of central respiratory drive, measured as a decrease in phrenic nerve activity. This finding is consistent with the results of a number of previous studies which have reported inhibition of respiration in response to electrical stimulation of the mesencephalon (Kabat, 1936; Baxter & Olszewskik 1955; Evans & Pepler, 1974; Coles, 1987).

The motivation for this study was to locate the region of the mesencephalon responsible for the post-hypoxic inhibition of respiration which we have previously reported (Gallman & Millhorn, 1988). If hypoxia were acting directly upon neurones of the mesencephalon, it could, theoretically, operate in at least two manners. First, hypoxia could directly inhibit tonically active mesencephalic neurones which normally potentiate some aspect of respiratory drive. The loss of this tonic facilitatory activity would be seen as a decrease in respiratory output. Alternatively, hypoxia could directly stimulate neurones in the mesencephalon. These neurones could, in turn, mono- or polysynaptically inhibit respiratory-related circuits in the caudal brain stem. If this were the case, one would expect to find mesencephalic neurones which, when stimulated, would cause phrenic nerve inhibition. We tested the hypothesis that stimulation of mesencephalic neurones could elicit inhibition of the phrenic nerve and found it to be true.

The assertion that post-hypoxic phrenic inhibition and phrenic inhibition following mesencephalic stimulation are expressions of the same mechanism is predicated upon the assumption that hypoxia can, in some way, excite neurones. There is, at present,

no evidence indicating that hypoxia directly excites mesencephalic neurones. However, such excitation has been found in other neuronal systems. Recent *in vitro* electrophysiological experiments in this laboratory have revealed a population of neurones within the confines of the nucleus of the solitary tract (NTS) of the medulla oblongata which are excited by hypoxia (Gallman, Dean & Millhorn, 1990). Schiff & Somjen (1985) have demonstrated that hippocampal neurones in slice preparations become hyperexcitable following exposure to hypoxia. This hyperexcitability lasts at least 45–55 min, which is consistent with both the duration of post-hypoxic inhibition, which lasted at least an hour (Millhorn *et al.* 1984; Gallman & Millhorn, 1988), and the duration of the longest periods of phrenic inhibition seen following EAA injection in the present study, which ranged from 20 to 40 min.

Electrical stimulations, such as those employed in the present study, are not intended to mimic any sort of physiological activation. They are used merely to determine which, of the range of possible respiratory responses, can be elicited from a given location. Evans & Pepler (1974), for instance, described nineteen different types of respiratory response during electrical stimulation of brain stem in rabbit. It was not our intention to produce a similar study in cat, but rather to locate likely target sites for subsequent EAA stimulation.

Discrete, unilateral EAA stimuli might not be expected to produce a response identical in magnitude or duration to the response elicited by exposure to hypoxia, which presumably is acting generally and bilaterally. Consequently, we did not limit ourselves to consideration of only long-lasting responses, nor did we anticipate that phrenic inhibition in response to discrete chemical injections made into the mesencephalon would approximate in magnitude the phrenic inhibition we have previously reported following systemic hypoxia. Surprisingly, we did find that, in some cases, the magnitude of the inhibition of phrenic nerve activity elicited by EAA injections was as great as that seen following hypoxia. While we rarely saw inhibitions lasting up to 1 h following these injections, a duration of 20 min was not uncommon. These durations, however, may be attributable to the time course of removal of the EAA, discussed in more detail below.

We often noted that it was possible to evoke a large response from a location which was only 1 mm distant from a site from which we could not evoke a significant response to injection. This observation suggests that significant spread of injected amino acid was less than 1 mm in any direction, and that discrete regions of the mesencephalon were responsible for the changes in phrenic activity. While we did not determine exactly the spread of amino acid we can estimate this from the theoretically derived equations and assumptions of Nicholson (1985), using case 2, i.e. bolus pressure injection of a substance into brain tissue (volume fraction, α, = 0·21), assuming the substance infiltrates the extracellular space. The initial radius, b, is given as:

$$b = (3U/4\pi\alpha)^{\frac{1}{3}},$$

where U = injected volume. For U = 100 nl, a volume larger than many of the injections in the present study, the initial radius is about 485 μm. Nicholson also addressed the question of diffusion over time. For a 100 nl injection, he estimated that the peak concentration 700 μm from the centre of injection would occur at slightly over 2 min and would be about one-tenth the concentration of the injectate.

Using eqn (15) of Nicholson as a rough approximation, the maximum concentration 1 mm from a 100 nl injection of 0·5 M-glutamate will be less than 20 mM.

Excitatory amino acids rather than electrical stimuli were primarily used in the present study, as this method has been shown to restrict the stimulation to cell bodies without activating axons of passage (Zieglgansberger & Puil, 1973; Goodchild *et al.* 1982). Nevertheless, one must exercise caution in interpretation of such injections. One problem (considered above) is the spread of injectate. A second consideration is the possibility of a depolarizing block (Curtis, Phillis & Watkins, 1960; Crawford & Curtis, 1964). EAA injections may, in fact, cause only a brief (< 1 min) activation of cells within the initial sphere of injection (i.e. within 500 μm of the centre of injection) followed by an extended (> 30 min) period of depolarizing block (Nicholson, 1985; Lipski, Bellingham, West & Pilowsky, 1988). There is also some indication that, while that somata of some neurones may depolarize to the point that they are unable to initiate action potentials, the axons of such neurones may, by electrotonic conduction of the same depolarization, be raised to threshold. Thus, instead of being temporarily silenced, these cells may be in a temporary state of extreme activation. Additionally, somata outside the initial sphere of injection (500–1300 μm from the centre) may be excited for a number of minutes (Lipski *et al.* 1988).

Two observations lead us to believe that we are able to report results which are due to activation of somata rather than depolarizing block. First, we and others (Kabat, 1936; Baxter & Olszewski, 1955; Evans & Pepler, 1974; Coles, 1987) have demonstrated with electrical stimulation that activation of these areas can lead to respiratory inhibition. Such stimulation can be due to activation of cell bodies in the area or to activation of axons of passage, but not to depolarizing block. The second observation is that we have demonstrated repeatability of the inhibitory responses, but only after 15 min. Injections occurring within 15 min of previous injections probably did cause a depolarizing block (Lipski *et al.* 1988) and were in every case unable to elicit a second inhibition. If the phrenic inhibition which we observed following first injections was, itself, due to depolarizing block of mesencephalic neurones (which theory demands that the same neurones supply a tonic excitation to respiratory drive), then repeat injections within 15 min should have intensified and/or prolonged the inhibition from the first injection by ensuring a prolonged depolarizing block. Since this was not the case, it is likely that the first injections excited cells and produced phrenic inhibition while hasty second injections at the same site pushed those cells to the point of blocking and the inhibition was lost.

The likelihood of a depolarizing block at the centre of an injection does, however, affect interpretation of the exact location of cell bodies stimulated. Thus, while we feel confident in stating that our results are due to activation of cell bodies of the ventromedial mesencephalon, it is premature to state that these cell bodies do (or do not) lie within the nearest anatomical landmark, the red nucleus.

In the present study, we have removed or controlled many inputs to the central respiratory control network. Arterial P_{CO_2} was controlled and peripheral chemoreceptor inputs were removed. The use of deep surgical anaesthesia reduced the influence of the higher brain. Consequently, we have been able to study a response which, in an intact or an awake adult animal, may be masked by other inputs to the

central respiratory network. Therefore, the physiological importance of such a mechanism may be realized only in very select circumstances. Inhibition of fetal respiratory movements in response to hypoxia (Boddy, Dawes, Fisher, Pinter & Robinson, 1974) may be such a circumstance, as may the secondary depressive component of the biphasic response to hypoxia often noted in neonatal animals (Cross & Oppe, 1952). Dawes *et al.* (1983) found that a suprapontine region was responsible for the hypoxia-induced apnoea in fetal lambs. Martin-Body & Johnston (1988) were able to abolish the depressive component of the respiratory response to hypoxia in neonatal rabbits by decerebration near the junction of the pons and mesencephalon while more rostral transection, which left the mesencephalon intact, was not effective. Hypoxic depression of respiratory frequency in awake adult rat has also been demonstrated, through similar transection experiments, to require the rostral pons and/or caudal mesencephalon (Martin-Body, 1988).

One important difference exists between other studies concerning hypoxic depression and those from this laboratory. While most studies have concentrated upon respiratory responses during hypoxia, we have focused our attention on responses which persist after return to normoxic or hyperoxic conditions. There is, however, no reason to believe that these are entirely different mechanisms. It is certainly likely that some mechanisms activated during hypoxia abate following the end of the hypoxic episode. Nevertheless, it is probable that any mechanism responsible for depression of ventilation following hypoxia is actually being expressed during hypoxia as well.

We have demonstrated that stimulation of neurones in the mesencephalon can result in inhibition of phrenic nerve activity. It appears that neurones within and/or dorsal and medial to the red nucleus would have been activated by our EAA injections. However, the exact identity and nature of the projecting neurones, the region to which these cells project and the neurotransmitter(s) involved are unknown at present.

Both the EAA-evoked inhibition reported here and the previously reported hypoxic depression of respiration often involved a decrease in the rate as well as the amplitude of phrenic bursts. As it is generally agreed that the anatomical substrate(s) for the respiratory rhythm generator reside at the level of the medulla (von Euler, 1983), a change in respiratory rate infers medullary involvement. Thus the dorsal respiratory group and the ventral respiratory group are possible targets for the mesencephalic cells which we have activated. It is unlikely that the dorsal respiratory group receives direct input inasmuch as no such pathway has been revealed by anterograde transport of tritiated leucine injected into around the red nucleus in cat (Edwards, 1972) or by retrograde transport of horseradish peroxidase injected into the dorsal motor nucleus of the vagus or the nucleus of the solitary tract in rat (Horst, Luiten & Kuipers, 1984). There does, however, appear to be a pathway running from the region dorsal and medial to the red nucleus to the nucleus ambiguus of the ventrolateral medulla (Horst *et al.* 1984).

There is a precedent for an inhibitory pathway from the red nucleus to nucleus ambiguus. Schmid *et al.* (1988) investigated the pathway by which electrical stimulation of the rubrospinal tract elicits inspiratory inhibition in rabbit and suggested that a population of bulbospinal inspiratory neurones were inhibited

monosynaptically by stimulation of the tract. The bulk of respiratory-related medullary units they studied were in the region of the ambigual complex. Electrical stimulation in mesencephalic rat has also proven to inhibit respiration (Coles, 1987). The location of this stimulus was described merely as 'ventrolateral... brain stem at the level of the superior colliculi'. An electrical stimulus to this region would quite likely have activated the rubrospinal tract.

There may also be evidence that a rubrobulbar branch of the rubrospinal tract is involved in mediation of hypoxic inhibition. Gluckman & Johnson (1987) were able to abolish hypoxic depression of breathing in fetal lambs by lesioning areas of the ventrolateral pons and mesencephalon. Only a very small bilateral region was common to all lesions which prevented the fetal hypoxic depression. This site, though not identified as such, appears to overlay the rubrospinal tract (Fig. 6, Gluckman & Johnston, 1987).

Thus, evidence is accumulating which indicates that a region of the caudal mesencephalon, possibly including elements of the red nucleus, is involved in hypoxia-induced inhibition of breathing. This inhibition is more likely to be directed towards the ventral than the dorsal respiratory group. The physiological significance of such an inhibitory mechanism may relate to fetal suppression of respiratory movements. The similarity between the apparent anatomical substrates for the fetal hypoxic depression and components of hypoxic inhibition seen in the adult suggests they may be one and the same.

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