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ALTERATION OF MEDULLARY RESPIRATORY UNIT DISCHARGE BY IONTOPHORETIC APPLICATION OF PUTATIVE NEUROTRANSMITTERS

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1 Cats with midcollicular decerebration were vagotomized, paralyzed and artificially ventilated. Phrenic nerve activity was recorded as an index of central respiratory rhythm. Medullary respiratory neurones and non-respiratory cells located in approximation to the ventral respiratory nucleus were tested for their responsiveness to iontophoretically applied γ-aminobutyric acid (GABA), acetylcholine (ACh) and glutamate.

2 GABA tended to inhibit, whereas ACh and glutamate excited activity both of respiratory and non-respiratory units. Some phase-spanning respiratory unit activities were converted to phasic discharge patterns linked to either inspiration or expiration concomitant with application of low GABA doses. Appropriate applications of GABA also resulted in a complete cessation of the respiratory or non-respiratory neuronal activities.

3 While application of ACh or glutamate induced continuous firing in phasic, phase-spanning respiratory neurones, the periodic discharge patterns of inspiratory or expiratory units was not altered by ACh or, in many instances, by glutamate. Only at high doses of glutamate was the phasic discharge of some inspiratory or expiratory units converted to tonic activity.

4 These observations suggest that strong inhibitory processes serve to maintain the phasic firing pattern of respiratory units. These data also support the concept that active-inhibitory phase-switching mechanisms serve to define respiratory rhythmicity.

Introduction

Neurones, whose pattern of activity is linked to some portion of the respiratory cycle, can be localized throughout the pons and medulla of cats (e.g. Cohen & Wang, 1959; Vibert, Bertrand, Denavit-Saubié & Hugelin, 1976). The linkage of these unit activities with the respiratory cycle is such that some units discharge only in phase with either inspiration or expiration (Cohen & Wang, 1959; Cohen, 1968; 1970; Bianchi, 1971; Bertrand, Hugelin & Vibert, 1974; Vibert *et al.*, 1976). Other units, designated as phase-spanning, exhibit activity coincidental with portions of both the inspiratory and expiratory phases (Cohen, 1968; 1970; Bertrand *et al.*, 1974; Mitchell & Berger, 1975). Phase-spanning respiratory units may exhibit phasic or tonic patterns of activity (Cohen, 1968, 1970; Bertrand *et al.*, 1974; Mitchell & Berger, 1975).

Inherent to the many theories (Cohen, 1970; Clark & Euler, 1972; Mitchell & Herbert, 1974; Bradley, Euler, Marttila & Roos, 1975; Mitchell and Berger, 1975; Berger & Mitchell, 1976; Feldman, 1976) for the neurogenesis of respiratory rhythmicity is a phase-switching mechanism by which one respiratory phase is terminated and the opposite phase started. Several investigators have recently proposed that active inhibitory mechanisms, arising from vagal afferent nerve fibres (Cohen, 1970; Clark & Euler, 1972; Bradley *et al.*, 1975; Mitchell & Berger, 1975) and/or from the pontile pneumotaxic centre (Cohen, 1970; Bertrand *et al.*, 1974; Euler & Trippenbach, 1976; Feldman, 1976) and/or from a 'switch neuronal pool' (Bradley *et al.*, 1975; Mitchell & Berger, 1975; Berger & Mitchell, 1976) serve to terminate inspiratory or

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expiratory respiratory unit discharge and, hence, promote a phase switch of the respiratory cycle.

The present studies were undertaken to examine some characteristics of these proposed mechanisms for respiratory rhythm generation. We reasoned that, if active inhibitory processes serve to terminate medullary respiratory unit discharge, it should be quite difficult, through pharmacological intervention, to convert these phasic respiratory unit activities to tonic discharge patterns. Our results obtained with the iontophoretic application of excitatory putative neurotransmitters support the concept that active inhibitory phase-switching mechanisms define respiratory rhythmicity.

Methods

Fourteen adult cats (3.5–5.0 kg) were anaesthetized with halothane during a midcollicular decerebration. Cannulae were placed in the trachea and in a femoral artery and femoral vein; a bilateral vagotomy at the mid-cervical level was also performed. Following a partial parietal–occipital craniotomy, the caudal portion of the cerebellum was removed in order to expose the floor of the IVth ventricle. C5 and/or C6 phrenic nerve rootlets were dissected in the neck and activity of these recorded as an index of central respiratory rhythm. The cat was then paralyzed with gallamine triethiodine and artificially ventilated. End-tidal CO_2 and O_2 partial pressures (P_{CO_2}, P_{O_2}) and arterial blood pressure were monitored as previously described (St. John, 1975). By adjusting appropriate gas mixtures delivered to the respirator intake port, the P_{CO_2} and P_{O_2} could be set at desired levels. In most studies, responses were evaluated under hyperoxic conditions (P_{O_2} > 500 mmHg) at constant P_{CO_2} levels (35–45 mmHg in different preparations). Rectal temperature was maintained at 38–40°C by a warm-water heating pad.

Records of single unit activity were made with five-barrel microelectrodes (3–6 μm tip diameter) as previously described (Kirsten & Sharma, 1976a, b). The recording barrel was filled with a 2% solution of pontamine sky blue in 4 M NaCl. Three of the four side barrels were filled with drugs; the fourth contained 4 M NaCl and was used as a current control and to balance drug ejecting and retaining currents. The micropipettes contained the following solutions: 0.4 M acetylcholine chloride (ACh, pH = 4.0, Sigma), 0.5 M γ-aminobutyric acid (GABA, pH = 4.0, Sigma), and 0.4 M L-glutamic acid (pH = 8.0, Sigma). Micropipettes were filled with the above solutions by the fibreglass filling technique (Tasaki, Tsukahara, Ito, Wayner & Yu, 1968).

Extracellular action potentials of single, well isolated neurones were detected from the central recording barrel (4 M NaCl, 2–5 MΩ), amplified and displayed on one beam of an oscilloscope. Amplified spike discharge was also routed through a level discriminator and ratemeter. To ensure that only action potentials of a constant amplitude were selected for study, the standard pulse output from the level discriminator was continuously monitored by display on a second oscilloscope beam. Neuronal discharge rate was plotted on a polygraph.

A modified Howland pump circuit (Geller & Woodward, 1972) was employed for iontophoresis of drugs. Ejecting and/or retaining currents could be applied to the drug barrels while the algebraic sum of these currents (but of opposite polarity) was passed through the micropipette balance barrel (4 M NaCl). Current could also be passed through the balance barrel to insure that responses were not due to electrotonic effects of the iontophoretic current.

The locations of units and/or microelectrode tracts were marked by passing 10 to 15 μA cathodal current through the recording barrel for 10 min resulting in the deposition of Pontamine blue in a localized spot (Boakes, Bramwell, Briggs, Candy & Tempesta, 1974). At the completion of all experiments, the animals were perfused with formalin and frozen sections (25–50 μm) of the brainstem cut from 24 to 48 h later. The localization of the dye spot served as a reference point for the estimation of each neurone studied. Following verification of the blue dye marks, the sections were stained by the Weil method and the recording sites referred to one of three sections taken at approximately 1.0 mm intervals.

In addition to anatomical locations, brainstem units were further defined as to their firing characteristics during one (or more) phases of the respiratory cycle (Cohen, 1968). Medullary respiratory units, concentrated in proximity to the obex, displayed phasic bursts of firing coinciding essentially with either inspiration, expiration or both respiratory phases (see Introduction).

Results

Those sites at which activity was recorded from inspiratory, expiratory and phase-spanning respiratory units, as well as from non-respiratory units, were concentrated in an area of the ventrolateral medulla approximating the nucleus ambiguus (Figure 1). The medullary respiratory units were thus localized within that area termed by others (Bianchi, 1971) as the ventral respiratory nucleus.

As indicated by the data of Table 1, a consistent depression of medullary neuronal discharge occurred when GABA was applied, whereas glutamate administration consistently resulted in neuronal excitation. Although responses to ACh were more mixed than

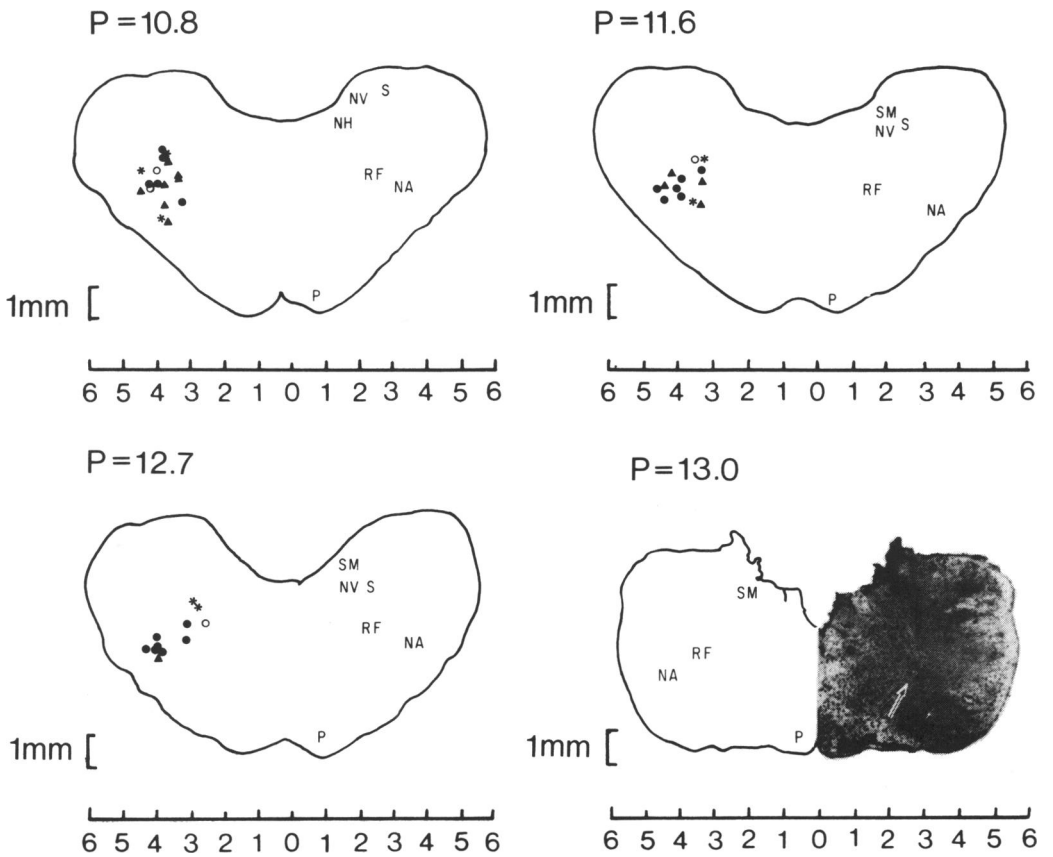

Figure 1 Localization within the medullary brainstem of units whose activities were monitored. The site at which inspiratory (●), expiratory (▲) and phase-spanning (○) respiratory units and nonspecified, non-respiratory units (*) were recorded, as defined by stereotaxic location of the microelectrode and/or location of the pontamine blue dye spots, were projected on the nearest rostral-caudal section to those noted (P = 10.8–12.7). (All units, regardless of location were drawn on the left side of the medulla.) P values are distances in millimeters caudal to the interaural stereotaxic zero (Snider & Niemer, 1961). Scales below each medullary section indicate the distances lateral to the midline in millimeters. The location of a pontamine blue dye spot is indicated by the arrow in the P = 13 section. NA = nucleus ambiguus, NH = nucleus of the hypoglossal nerve, NV = dorsal nucleus of the vagus, P = pyramidal tract, RF = reticular formation, S = solitary tract, SM = medial nucleus of the solitary tract.

Table 1 Qualitative response of inspiratory (I), expiratory (E) and phase-spanning (P-S) medullary respiratory units and non-specified, non-respiratory (N) medullary units to iontophoretic application of glutamate (Glu), γ-aminobutyric acid (GABA), and acetylcholine (ACh)

Neuronal type	*Total units*	*GABA*			*Glu*			*ACh*		
		+	−	=	+	−	=	+	−	=
I	33	0	15	1	21	0	0	12	2	2
E	21	0	8	1	15	0	0	10	1	4
P-S	9	0	3	0	5	0	0	4	1	1
N	28	0	21	0	22	0	0	13	7	0

In each column, the number of units which exhibited an increase (+), a decrease (−) or no change (=) in peak firing frequency are indicated. Not all units were tested with all three compounds.

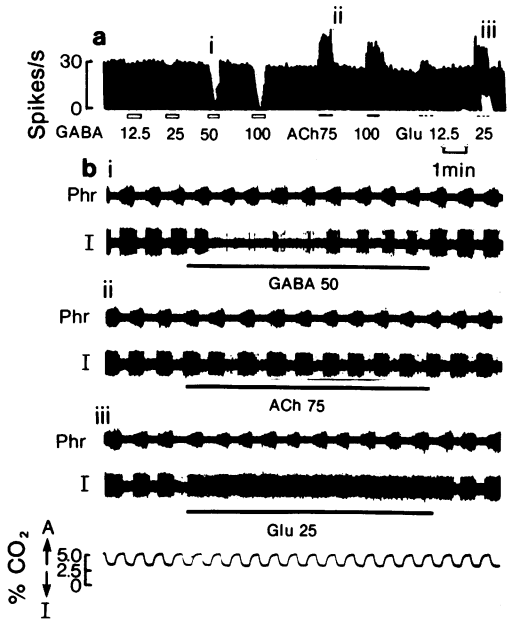

Figure 2 Response of spontaneously discharging inspiratory neurone to iontophoretically applied γ-aminobutyric acid (GABA), acetylcholine (ACh) and glutamate (Glu). (a) Polygraph recording of neuronal discharge rate (see Methods) obtained before, during, and after neurotransmitter applications. The applied ejection currents (nA) of each neurotransmitter and the period of application (GABA □, ACh ——, Glu – – –) are shown below the polygraph record. Note that, before neurotransmitter application, the neuronal discharge rate varied from 0 to approximately 30 spikes/second. These discharge rates reflect the phasic discharge pattern of the inspiratory unit. An inhibiton of neuronal firing was obtained with application of GABA. This inhibition was most marked at GABA ejection currents of 50 and 100 nA. In contrast, applications of ACh (75 and 100 nA) or glutamate (12.5 and 25 nA) increased the discharge frequency within an inspiratory burst. (b) Records of phrenic nerve (Phr) and inspiratory unit (I) activities obtained during those periods indicated by i, ii and iii in (a). (i), GABA application caused a decrease in the number of spikes per inspiratory burst. Some recovery of the inspiratory unit discharge was noted during the latter period of drug application. (ii) ACh increased the peak spike frequency and also the burst duration of the inspiratory unit. (iii) Application of glutamate, at an injection current of 25 nA, converted the phasic discharge pattern to tonic activity. A typical tracing of inspired (I) and end-tidal (A) CO_2 levels is indicated during the glutamate application. In (i), (ii), and (iii) drugs were applied for 30 s periods (indicated by bars); the injection current of each drug is given in nanoamperes (nA).

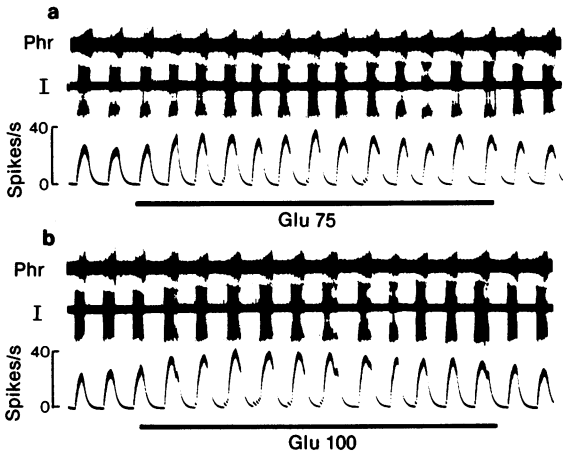

Figure 3 Alteration in inspiratory unit discharge by glutamate. (a & b) The phrenic nerve (Phr), inspiratory neurone (I) and rate-meter record (in spikes/s) during 30 s pulses of glutamate (Glu 75 and 100 nA). Note the increases in peak firing frequency and duration of inspiratory unit discharge.

those of GABA or glutamate, most units exhibited increases in activity following ACh application. Statistical analysis, with a χ^2 test, indicated no significant ($P > 0.05$) qualitative difference between the various neuronal types in their response to GABA, glutamate or ACh (Table 1). However, the specific characteristics of the changes in neuronal activity did differ depending upon neuronal type, the agent applied, and the time after drug application at which responses were evaluated.

Some differences in response to iontophoretic drug application are illustrated in Figure 2. Thus, while it was observed that application of 50 nA of GABA (Figure 2, ai, bi) resulted in an essentially complete cessation of the inspiratory unit activity, the discharge of this unit progressively increased with time towards control rates despite continuing GABA administration. This spontaneous reversal of the GABA-induced depression, which may be the result of rapid adaptation to the high GABA doses (Kelly & Krnjević, 1968), was frequently observed for all respiratory neuronal types though not for non-respiratory units. It is of interest to note that, in confirmation of other investigators (Denavit-Saubié & Champagnat, 1975), application of lower GABA doses was effective in converting the activity of some phase-spanning respiratory units to a phasic discharge essentially linked to either inspiration or expiration.

ACh, when applied at ejection currents of 50 to 150 nA, increased the peak firing frequency of 72%

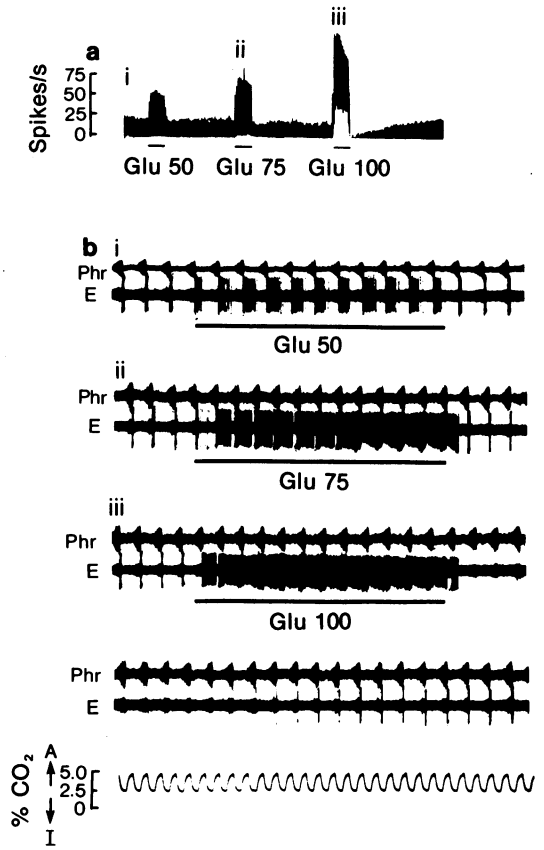

Figure 4 Dose-related excitatory response of an expiratory neurone to iontophoretically administered glutamate. (a) Polygraph recording of neuronal discharge rate (See Methods) obtained before, during, and after application of glutamate at 50, 75 and 100 nA. Each dose was administered for 30 s as indicated by the lines below the polygraph record. Note the increase in peak firing frequency during each glutamate application. (b) Records of phrenic nerve (Phr) and expiratory unit (E) activities obtained during those periods indicated by (i), (ii) and (iii) in (a). The 30 s periods of glutamate application are indicated by the line below each pair of tracings. In tracings (i), (ii), and (iii), note that glutamate application increased both the peak firing frequency and the burst duration of the expiratory unit. Note in (iii) (upper pair of tracings) that despite the marked increase in firing frequency (Glu 100), the phasic discharge of the expiratory unit was maintained. The lower pair of tracings in panel (iii) are continuous with the upper pair of tracings and reveal the inhibition of expiratory neuronal activity which followed glutamate application at 100 nA. A typical record of inspired (I) and end-tidal (A) CO_2 levels obtained concomitant with the lower pair of panel (iii) tracings is indicated.

of the respiratory units tested. Despite this increased activity, inspiratory (Figure 2) and expiratory units maintained their phasic discharge characteristics. This inability of ACh to convert phasic discharges to patterns of continuous firing was characteristic of all inspiratory and expiratory units examined. In contrast, phase-spanning units, having discontinuous patterns of activity, exhibited an essentially tonic discharge after application of ACh. Results obtained following application of glutamate both confirmed and extended the findings with ACh.

Glutamate (10–100 nA) augmented the activity of all neurones tested. As illustrated in Figure 2biii, glutamate converted some phasic neuronal discharge patterns to tonic activity. This alteration to a tonic discharge was obtained for all (n = 5) of the phase-spanning units examined. However, 60% of the inspiratory (e.g. Figure 3) and 43% of the expiratory (e.g. Figure 4) units maintained their phasic discharge characteristics despite a several-fold increase in firing frequency at high glutamate ejection currents. Thus, although the peak firing frequency of the expiratory unit of Figure 4 was increased by approximately 275% at the highest glutamate dose, a cut-off of discharge still was evident (Figure 4biii) during one portion of the respiratory cycle. This unit of Figure 4biii was also of interest in that, upon termination of glutamate ejection (100 nA), a complete cessation of activity was evident for several respiratory cycles; later, neuronal activity slowly returned. A similar post-glutamate inhibition of discharge was observed for 30% of all respiratory units examined, though not for any non-respiratory units. This post-glutamate inhibitory response was probably due to the strong depolarization associated with sodium ion influx (Koike, Mano, Okada & Oshima, 1972) and subsequent activation of an electrogenic sodium pump (Zieglgänsberger & Puil, 1973).

Discussion

The activity of single units in decerebrate cats was examined for their responses to iontophoretically applied GABA, ACh and glutamate. These cells were characterized as either inspiratory, expiratory, phase-spanning or non-respiratory based upon the time relationship of the burst discharge to that of phrenic nerve activity. In agreement with previous investigations of brainstem units (Avanzino, Bradley & Wolstencroft, 1966; Obata, Ito, Ochi & Sato, 1967; Kirsten & Sharma, 1976b) the predominant action of iontophoretically applied GABA was inhibitory while ACh and glutamate produced excitatory responses on identified respiratory or non-respiratory units in the area of the obex.

By direct statistical comparison (χ^2 test, see Results) the receptive properties of the neuronal membrane of respiratory neurones are similar to those of non-respiratory units. While the potencies of the two putative amino acid neurotransmitters appeared greater than that of ACh, no attempt was made to replicate the differences in sensitivities between respiratory and non-respiratory units reported previously (Denavit-Saubié & Champagnat, 1975). Responses of the respiratory units tested with ACh were considerably more uniform than observed in an earlier study (Salmoiraghi & Steiner, 1963) where the discharge of only two of 34 respiratory units were altered by ACh whereas in the present study 72% of the respiratory units were excited by ACh. This discrepancy may be due to the more diffuse location of cells (4–5 mm rostral to obex) in the previous study as compared with our recordings within or near the nucleus ambiguus.

Results obtained in this study suggest that active inhibitory processes serve to maintain the phasic discharge characteristic of respiratory units. Thus, ACh application was completely ineffective in converting the phasic firing pattern of inspiratory or expiratory units to a tonic discharge. Moreover, iontophoresis of glutamate, even at the highest dose levels (100 nA), did not effect this conversion in 45% of these phasic units.

Two further observations support this concept of a strong synaptic inhibition during a respiratory neurone's silent period. In a few studies ($n = 2$), normally silent respiratory neurones could be made to fire synchronously with expiration during application of glutamate. Most likely, these cells were undergoing subthreshold cyclic changes in membrane potential (Richter, Heyde & Gabriel, 1975) and glutamate increased the magnitude of these spontaneous rhythmic depolarizations (Mitchell & Herbert, 1974) or depolarized the cell sufficiently to reach the threshold for spike activation. Secondly, while only glutamate was capable of altering the phasic discharge characteristics of inspiratory or expiratory units, both ACh and glutamate converted the discontinuous activity of phase-spanning respiratory units to a continuous discharge. Also we and others (Cohen & Wang, 1959) have observed that phase-spanning units periodically exhibit one or more spikes during the normally silent period between bursts. Both of these observations imply that the inhibition impinging upon phase-spanning units is less than that impinging upon inspiratory or expiratory units.

In summary, we believe that the major observation of the studies described here was the maintenance of a periodic discharge by most inspiratory and expiratory units despite a several-fold increase in discharge frequency resulting from iontophoretic application of excitatory putative neurotransmitters. These results are consistent with the hypothesis recently suggested by others (Bradley *et al.*, 1975; Richter *et al.*, 1975) that strong inhibitory mechanisms may act to maintain the periodicity of respiratory units. Moreover, these data provide further experimental support of the model system for respiratory rhythmicity advanced by Euler and his colleagues (Clark & Euler, 1972; Bradley *et al.*, 1975; Euler & Trippenbach, 1976). A fundamental component of this model system maintains that active-inhibitory phase-switching mechanisms may serve to define respiratory periodicity.

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