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RESPIRATORY RHYTHM GENERATION IN THE *IN VITRO* BRAIN STEM–SPINAL CORD PREPARATION OF THE NEONATAL RAT

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SUMMARY

1. An *in vitro* preparation was described for studying electrical activity of mammalian brain stem and spinal cord.

2. The brain stem and the spinal cord were isolated from 0–4-day-old rats, placed in a bath and perfused with modified Krebs solution. Various reflex responses could be recorded from cranial nerves by stimulation of other cranial nerves. The preparation was viable for more than 7 h.

3. Spontaneous periodic neural activity could be recorded from phrenic, hypoglossal and other spinal nerves.

4. The periodic discharges of phrenic nerves are synchronized with those of ventral roots C4 and the upward movements of the thorax which was isolated together with the spinal cord.

5. The rhythm of periodic activity seems to be generated in the brain stem.

6. The periodic activity was enhanced by perfusion with low pH solution and depressed by high pH solution. It was markedly depressed by opioid compounds such as enkephalin.

7. It is suggested that this periodic activity corresponds to the respiratory rhythm generated in the brain stem of intact animals.

8. The present preparation may be valuable for elucidating cellular mechanisms of generation and control of respiratory rhythm in the mammalian central nervous system.

INTRODUCTION

Brain slice preparations have proved to be useful experimental tools for physiological and biochemical investigations of the mammalian central nervous system (Andersen, 1981). This method, however, has an inherent disadvantage, in that the slice preparations must be made thin enough to allow sufficient oxygen supply to the tissues. Thus, the majority of input and output fibres to the neurones are necessarily severed (Andersen, 1981). Moreover, it is difficult to make preparations containing many intact brain areas which together carry out higher physiological functions.

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Otsuka & Konishi (1974) introduced the isolated spinal cord preparation of the new-born rat. This is a unique *in vitro* preparation of whole mammalian spinal cord, in which activities of many neural networks are kept functioning. This technique has been extended and a method of isolating the brain stem and spinal cord has been developed (Suzue, 1983). In this latter preparation some of the physiological functions are maintained; spontaneous periodic neural activity as well as the reflex responses evoked by electrical stimulation of cranial nerves were observed. The results of the present study suggest that this spontaneous periodic activity may correspond to the respiratory rhythm generated in the intact animals. The *in vitro* brain stem–spinal cord preparation offers an opportunity for studying some higher physiological functions in the central nervous system; this has not been possible using slice preparations. Part of this work has been reported briefly (Suzue, 1983).

METHODS

Isolation and perfusion of brain stem–spinal cord preparation

Wistar rats from birth to 4 days of age were used. Under deep ether anaesthesia, the head and vertebral column were isolated and placed in a dissection chamber filled with the modified Krebs solution (for composition see below). The skin covering the skull and the occipital and frontal bone were cut off with scissors. The cerebrum was quickly removed by transecting at the intercollicular level, and the cerebellum was also removed. The above procedures were completed within 2–3 min and the anaesthesia lasted much longer than this period. Therefore there is no possibility of the animals suffering during the dissection or the experiments. More of the skull and vertebrae was removed and as much as possible of the cranial and the spinal nerves was retained. The preparation was then placed in a 0·5–2 ml bath and perfused at a rate of 4–5 ml/min with modified Krebs solution (NaCl, 123 mM; KCl, 5·0 mM; KH_2PO_4, 1·23 mM; $CaCl_2$, 2·4 mM; $MgSO_4$, 1·3 mM; $NaHCO_3$, 26 mM; glucose, 30 mM) equilibrated with 95 % O_2–5 % CO_2 gas. A few experiments were performed with artificial cerebrospinal fluid (Suzue & Jessell, 1980) containing 30 mM-glucose. Essentially the same results were obtained in both kinds of solutions. The temperature of the perfusion medium was kept at 27 °C. This temperature was adopted because the amplitude of the reflex responses in the preparation was largest and best maintained. At higher temperatures, the preparation tended to deteriorate probably because of the increased O_2 and glucose consumption. The same temperature has been used for isolated neonatal rat spinal cord preparations (Otsuka & Yanagisawa, 1980; Suzue & Jessell, 1980).

Recordings

Suction electrodes were used for stimulation of dorsal roots and cranial nerves. For extracellular recording from cranial nerve roots, ventral roots and phrenic nerves, tightly fitting glass capillary suction electrodes (inner diameter of the tip 50–130 μm) were used (Otsuka & Konishi, 1974; Otsuka & Yanagisawa, 1980). The electrodes were filled with physiological solution and connected through Ag–AgCl wires to pre-amplifiers. The responses evoked by stimulation of the trigeminal nerve were recorded from cranial nerves and cervical ventral roots and displayed on an oscilloscope. When the spontaneous periodic activity was recorded from ventral roots C4 and C5, the phrenic nerve and the XIIth cranial nerve, the output of the pre-amplifier was fed to a potentiometric pen recorder (Gould 2200) via a simple RC high-pass filter (time constant 1 s). The high-pass filter was used to diminish the slow drift of the extracellular potentials in Figs. 2, 3 and 4. The amplitude of the neural activity was slightly distorted because of the long response time in the pen recorder.

In some experiments respiration-like movements of the thorax were examined, and for this purpose brain stem–spinal cord preparations with attached unilateral ribs and intercostal muscles were made. The vertebral column and the contralateral ribs were removed as much as possible to allow easy access of oxygen to the spinal cord. The preparations were then firmly fixed at ribs Th1–Th3 to the rubber-layered bottom of the perfusion bath with stainless-steel needles. The respiratory movements were recorded by connecting a probe of force displacement transducer to the distal end of a rib. Drugs were dissolved in the modified Krebs solution and applied by perfusion.

RESULTS

Viability of the brain stem–spinal cord preparation of the new-born rat

The viability of the neural preparation can best be substantiated by the reflex activity which was retained. Fig. 1 shows responses of the preparations to a single shock stimulation of trigeminal nerve. Responses were recorded extracellularly from some cranial nerves (IX–XII) and cervical ventral roots. These responses could be

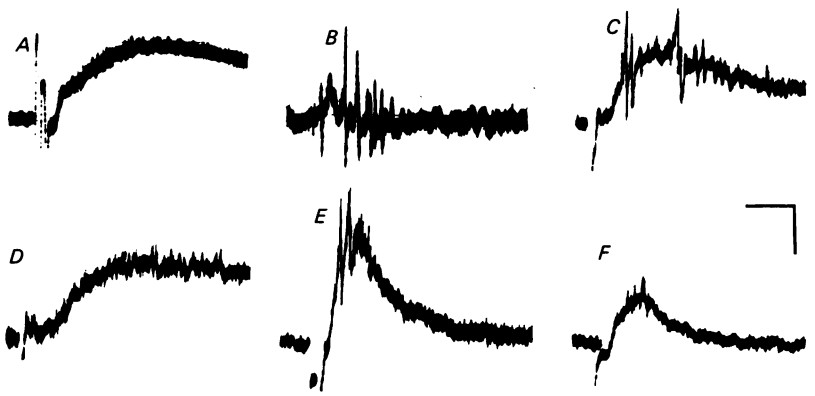

Fig. 1. Reflex responses elicited in various cranial nerves and spinal ventral roots in response to a stimulus delivered to the ipsilateral Vth cranial nerve in the isolated brain stem–spinal cord preparation of the neonatal rat. The records in *A* and *B* were obtained from nerve bundles including the IXth, Xth and XIth cranial nerves in a preparation from a 2-day-old rat. The record in *A* was obtained from a nerve bundle located in a more rostral part of brain stem surface than the nerve bundle from which the record in *B* was obtained. The record in *C* was obtained from the spinal root of the XIth nerve of a 0-day-old rat. The records in *D*, *E* and *F* were each obtained from the XIIth nerve, ventral root C1 and ventral root C2 of a 1-day-old rat. Calibration 200 μV for *A*, *B* and *C*; 100 μV for *D*, *E* and *F*; 40 ms for *A*, *C*, *D*, *E* and *F*, and 20 ms for *B*.

observed for at least 7 h after isolation without any significant sign of deterioration. Since these evoked potentials were abolished almost completely after perfusion with Ca^{2+}-free solution (Ca^{2+} 0 mM, Mg^{2+} 3 mM), they seem to represent mainly reflex activities involving synaptic transmission. Judging from the fact that the reflex potentials can be stably recorded for several hours, the neurones and neural circuits seem to be kept in a fairly good condition in the present preparation. More details are published elsewhere (Suzue, Murakoshi & Tamai, 1983).

Respiration-like spontaneous neural activity in the brain stem

In addition to the stimulus-induced passive responses, it was found that the brain stem–spinal cord preparation retains the ability to produce spontaneous and rhythmical neural activity. When a brain stem–spinal cord preparation with part of the thorax attached was prepared and perfused with Krebs solution, spontaneous synchronous contraction of the intercostal muscles, i.e. respiration-like rhythmic movement of the thorax, was observed. This is seen in Pl. 1 which shows three photographs of the preparation taken successively. Fig. 2 shows the periodic

contraction of intercostal muscles (and other muscles which move thorax) by recording the tension which moves the ribs from a caudal to a rostral direction. Synchronously with the contraction of these inspiratory muscles, the depolarization of motoneurones was recorded extracellularly from the bilateral C4 (Fig. 2*A*) and C5

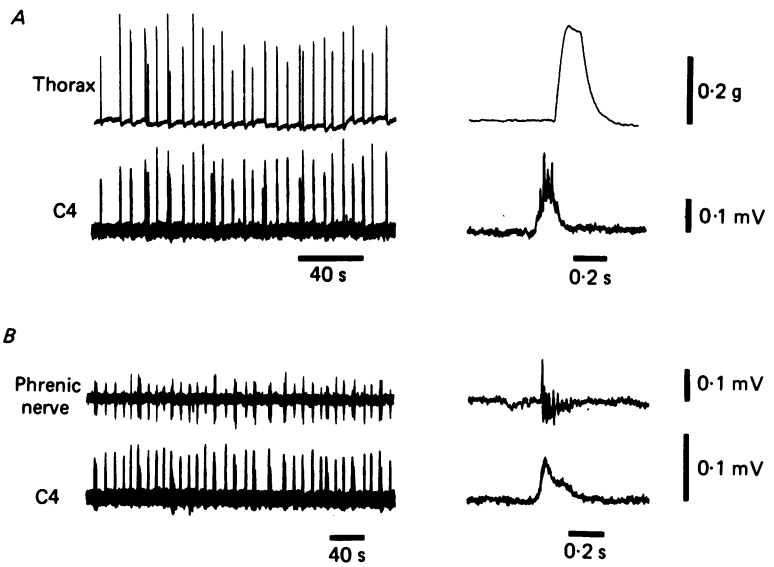

Fig. 2. Synchronization of the movement of the thorax with neural electrical activity of ventral root C4 and the phrenic nerve. *A*, simultaneous recordings of movement of thorax (upper traces) and extracellular potentials of ventral root C4 (lower traces) from a 0-day-old rat preparation. Movement of the thorax produced by the contraction of respiratory muscles was monitored by recording the tension from the distal end of the rib (right Th7). Upward deflexion represents the upward movement of the thorax. Extracellular potentials of the left ventral root C4 were recorded using a suction electrode. Positivity upwards. *B*, simultaneous recordings of extracellular potentials from the phrenic nerve and ventral root C4. Records obtained from the right phrenic nerve and left ventral root C4 of a 0-day-old rat. Recordings with expanded time scale are shown in the right column of each recording.

ventral roots. Since it is known that in the rat the phrenic nerves are derived from ventral roots C4 and C5, the depolarization recorded from these cervical ventral roots is likely to reflect the activity, i.e. action potentials and excitatory post-synaptic potentials, of the phrenic motoneurones. In fact, in other experiments spontaneous periodic discharges that were synchronized with the spontaneous depolarization of ventral root C4 were observed from the contralateral phrenic nerve (Fig. 2*B*). Thus in this preparation the firing of the phrenic nerves occurred synchronously with the contraction of the external intercostal and other inspiratory muscles which moved the thorax upward, just in the same way as is observed in intact animals during inspiration. It seems possible, therefore, that the rhythmical activity of brain stem–spinal cord preparations as shown in Fig. 2 corresponds to the activity of the respiratory rhythm generated in the central nervous system of the intact animals. This possibility was further examined in the following experiments.

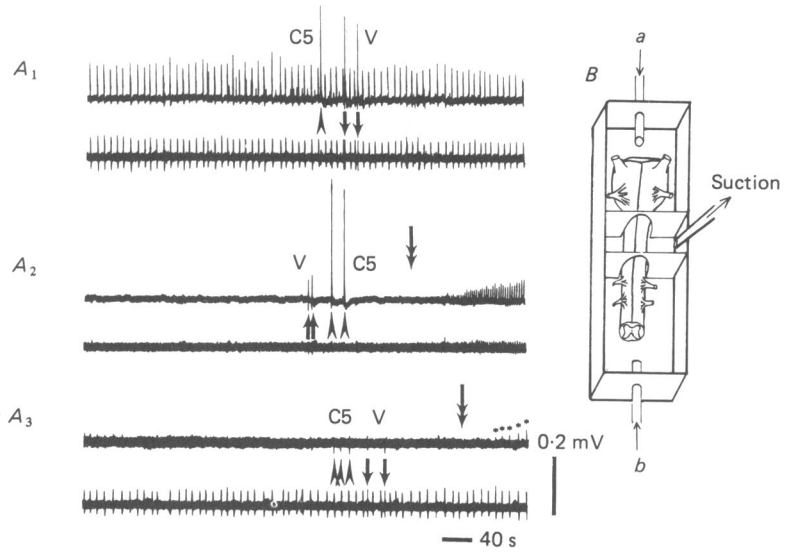

Fig. 3. The effect of Ca^{2+}-free medium on the spontaneous periodic neural activity. The brain stem and the spinal cord were separately perfused. *A*, simultaneous recordings were made extracellularly from the left ventral root C5 (upper trace) and the right XIIth cranial nerve (lower trace) using suction electrodes. Arrow and arrowhead indicate respectively single shock stimulation of the right Vth cranial nerve (V) and the left dorsal root C5 (C5). A_1, both the brain stem and the spinal cord were perfused with normal medium. Responses of the XIIth cranial nerve induced by stimulation of the Vth cranial nerve were of almost the same size as the spontaneous potentials, and they are seen as the deflexions under arrows (A_1 and A_3). A_2, records obtained 25 min after introduction of Ca^{2+}-free medium to the brain stem compartment. The spinal cord compartment was perfused continuously with normal medium. At the time indicated by the double arrow, normal medium was re-introduced to the brain stem compartment. A_3, records obtained 9·5 min after introduction of Ca^{2+}-free medium to the spinal cord compartment. Normal solution was re-introduced to the spinal cord compartment at the time indicated by the double arrow. Throughout this period the brain stem compartment was perfused with normal medium. Note the gradual recovery of spontaneous periodic neural activity with high frequency in A_2 and with normal frequency in A_3 (indicated by * in the upper trace). Records in A_1, A_2 and A_3 were obtained from a single preparation of a 2-day-old rat with a time interval of 64 min between A_1 and A_2 and 47 min between A_2 and A_3. *B*, schematic diagram of the chamber used for the separate perfusion of the brain stem and the spinal cord. The brain stem and the spinal cord were perfused with the medium introduced from tube *a* and tube *b* respectively.

The origin of the periodic activity

Previous transection experiments performed *in vivo* revealed that the respiratory rhythm is generated in the lower brain stem (Lumsden, 1923; for reviews see Wyman, 1977; Cohen, 1979). In the present preparation, by perfusing the brain stem and the spinal cord separately it was possible without damaging the preparation to show that the rhythm is generated in the brain stem. The brain stem–spinal cord preparation was placed in a long chamber (Fig. 3*B*). The perfusion solutions flowed into the chamber from both ends through two independent perfusion systems (*a* and *b* in Fig. 3*B*). The chamber was divided into three compartments by putting thin plastic

partitions at the levels of C2 and the lower part of the brain stem. Solution entering from both ends flowed into the central compartment through the gaps between the partitions and the surface of the preparation. A thin stainless tube was set in the central compartment to suck up the solution. In this way the brain stem and the spinal cord could be separately perfused.

When extracellular recordings were made from ventral root C5 and the XIIth cranial nerve and both the brain stem and the spinal cord were perfused with normal Krebs solution, well synchronized periodic activity was observed in both nerves (Fig. $3A_1$). Phasic discharges of the XIIth cranial nerves synchronized with the rhythm of respiration, have already been demonstrated *in vivo* (Lowe & Sessle, 1973; Sauerland & Mitchell, 1975; Hukuhara, 1976). Under normal conditions, reflex potentials were recorded from the XIIth cranial nerve and ventral root C5 after stimulation of the trigeminal nerve, and also from ventral root C5 after stimulation of dorsal root C5 (Fig. $3A_1$). When the synaptic transmission in the brain stem was depressed by introducing Ca^{2+}-free solution (Ca^{2+} 0 mM, Mg^{2+} 3 mM) only to the compartment containing the brain stem, periodic electrical activity of the XIIth nerve disappeared within 20 min (Fig. $3A_2$). The reflex responses in the brain stem, i.e. the reflex between the Vth and the XIIth cranial nerves, were almost abolished under this condition. It should be noted that the periodic activity recorded from the spinal nerve (C5) was also abolished even though synaptic activity in the spinal cord was not impaired, rather it was facilitated (Fig. $3A_2$). On the other hand, when only the spinal cord was perfused with Ca^{2+}-free solution, the effect was limited to the spinal cord. Although the periodic activity recorded from ventral root C5 disappeared within 8 min, the rhythmic activity recorded from the XIIth cranial nerve remained unchanged both in amplitude and frequency (Fig. $3A_3$). These results suggest that the spontaneous periodic rhythm is generated in the brain stem. The effect of Ca^{2+}-free solution was reversible (Fig. $3A_2$ and A_3).

When Ca^{2+}-free solution was introduced to the compartment containing the brain stem, during the first several minutes the frequency of the periodic rhythm increased (from 0·1 to 0·33 Hz), while the amplitude of the periodic activity gradually decreased. When the brain stem was perfused with normal Krebs solution after prolonged exposure to Ca^{2+}-free medium, periodic activity of high frequency and of small amplitude reappeared first, and then the amplitude gradually became larger and the frequency lower (Fig. $3A_2$). Thus, low Ca^{2+} medium increased the frequency of the periodic rhythm.

The effects of pH on the periodic neural activity

It has been shown by *in vivo* experiments that perfusion of ventral surface of the medulla with acidic fluid stimulates respiration (Loeschcke, 1974; Schlaefke, 1981). Since the chemosensitive structures in the ventral surface of the medulla and the area containing the respiratory rhythm generator are both retained in the present brain stem–spinal cord preparation, it is expected that the presumed respiratory rhythm will possess a sensitivity to pH changes. As shown in Fig. $4A$, when the preparation was perfused with a solution whose pH was elevated to 8·8 by adding NaOH (5 mM), the generation of the periodic activity recorded from ventral root C4 was markedly depressed, and this effect was reversed when the preparation was perfused again with

normal Krebs solution (pH 7·2). On the other hand, the frequency of the spontaneous activity was markedly increased when the pH of the perfusion medium was lowered to pH 6·8 by adding HCl (5 mM). Similar effects on the spontaneous periodic activity were observed when the pH was varied by replacing $NaHCO_3$ in the medium with NaCl at constant CO_2 gas concentration or by changing the CO_2–O_2 ratio of the gas mixture with which the perfusion medium was equilibrated (S. Tamai & T. Murakoshi unpublished observations).

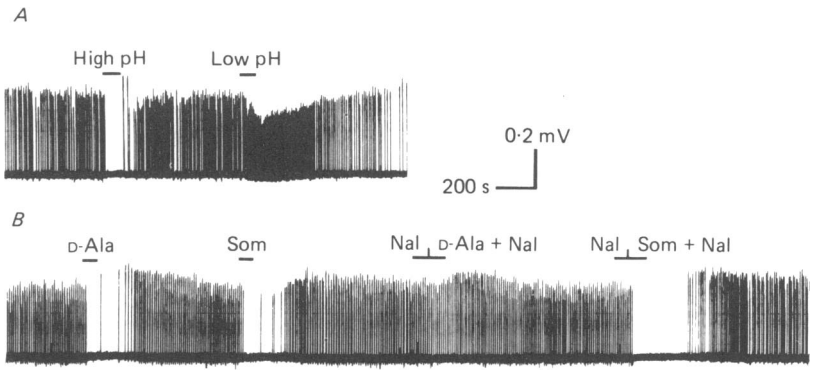

Fig. 4. Effect of pH and peptides on the spontaneous neural activity recorded from ventral root C4 of a 0-day-old rat. *A*, Krebs solutions of pH 8·8 (high pH) or pH 6·8 (low pH) were applied during the periods indicated by horizontal bars. *B*, [D-Ala²]-Met-enkephalinamide (D-Ala, 0·2 μM) and somatostatin (Som, 1 μM) were applied in the absence and in the presence of naloxone. In the latter cases, solutions containing peptides and naloxone (Nal, 0·8 μM) were applied after prior perfusion with the solution containing naloxone (80 s, 0·8 μM).

The effects of opioid compounds, somatostatin and trans-π-oxocamphor on the periodic neural activity

Since opioid compounds such as morphine are known to depress respiration (Pentiah, Reilly & Borison, 1966; Flórez, McCarthy & Borison, 1968), their effects on the spontaneous neural activity were tested. When the brain stem–spinal cord preparation was perfused with opioid peptides such as [D-Ala²]-Met-enkephalinamide (0·2 μM) or Met-enkephalin (1 μM), the frequency of spontaneous neural activity recorded from ventral root C4 was markedly reduced in a reversible manner (Fig. 4*B*). That the action of these peptides was mediated by opiate receptors was confirmed by the fact that the effects were reversed by naloxone (0·8 μM), an antagonist of opiate receptor (Fig. 4*B*). Somatostatin (1 μM) also reversibly reduced the frequency of periodic activity, but in contrast to the action of enkephalins this action was not antagonized by naloxone (0·8 μM; Fig. 4*B*).

Trans-π-oxocamphor (10^{-4} g/ml) which was used as a respiratory stimulant (Kumagai, Sakai, Hukuhara, Saji, Nakanishi, Sawabe & Nakazawa, 1964) increased the frequency of the spontaneous neural activity. NaCN at concentration of 0·1 mM also increased the frequency of the spontaneous neural activity.

DISCUSSION

The present results show that complicated functions of the brain stem, such as the rhythm generation, can be maintained *in vitro*. Since various activities can be observed for a long period in the present brain stem–spinal cord preparation, this *in vitro* preparation seems useful for the physiological, pharmacological and biochemical investigations of the central nervous system, especially when the higher physiological functions depending on several brain areas are to be examined.

Spontaneous activity with regular intervals could be recorded from the phrenic, hypoglossal and other spinal nerves in the present preparation. The evidence that this periodic activity corresponds to the respiratory rhythm generated in intact animals is as follows. (1) The contractions of the inspiratory muscles of the thorax (i.e. external intercostal muscles) are well synchronized with the discharges of phrenic nerves and the contraction of the diaphragm. (2) The spontaneous rhythm seems to be generated in the brain stem. (3) The periodic activity was enhanced when the pH of the perfusion medium was lowered and depressed when the pH was raised. (4) The frequency of the rhythm was markedly reduced by opioid compounds. These characteristics of the periodic activity in this *in vitro* preparation are similar to those of the respiratory rhythm generated *in vivo* (Pentiah *et al.* 1966; Flórez *et al.* 1968; Cohen, 1979; Schlaefke, 1981). Similar periodic neural activity has been observed in an *in vitro* preparation of the fish central nervous system (Rovainen, 1977).

There are some significant differences between the periodic rhythm of the present preparation and normal respiratory rhythm in the intact animal. First, the frequency of the spontaneous rhythm in the present preparation was low (0·1–0·2 Hz) as compared with the normal respiratory rate of intact neonatal rats (about 1·5 Hz), and one cycle of the rhythm is composed of a short inspiratory phase (when the phrenic nerve discharges) and a prolonged expiratory phase (when the phrenic nerve is silent). Because of this unique temporal relation between the durations of 'inspiratory' and 'expiratory' phases, the periodic rhythm may be correlated to gasping rather than the normal respiratory rhythm. The low frequency of the rhythm in the present preparation may be at least partly attributed to the absence of the afferent inputs; elimination of inputs from the periphery is known to reduce the frequency of respiration (Cohen, 1979). Furthermore, the relatively insufficient supply of oxygen to some part of the nervous tissue may also contribute to the lowering of the frequency of the periodic rhythm. Another difference to be noted is the response of periodic activity to the pH shift. In *in vivo* experiments performed in the cat, perfusion of the ventral surface of the medulla with low pH solution augmented ventilation mainly by increasing the tidal volume (Loeschcke, 1974; Schlaefke, 1981). In the present experiments, the most significant change of periodic activity after perfusion with low pH solution was the increase of the frequency, whereas the amplitude of the depolarizing potentials in ventral roots C4 and C5, which parallels the activity of the phrenic nerve, was not increased. However, direct comparison of the results of *in vivo* and *in vitro* experiments is difficult because of the many differences in the experimental conditions and experimental animals. In the present experiments, pH change was produced by bath application of solutions with varied pH. Therefore, the effect of pH change on some areas other than the chemosensitive

areas on the ventral medullary surface might have also contributed to the results. Further experiments using a more refined technique for local application of solution are required.

Among many neuropeptides, opioid peptides and somatostatin have been shown to have depressant actions on neurones of the mammalian central nervous system (Suzue, Yanaihara & Otsuka, 1981). In the present experiments, low concentrations of these peptides were shown to reduce the frequency of the periodic neural activity. These depressant actions of the peptides are interesting in view of the immunohistochemical evidence for the presence of a high density of enkephalin-like and somatostatin-like immunoreactivity in the nucleus tractus solitarius (Sar, Stumpf, Miller, Chang & Cuatrecasas, 1978; Finley, Maderdrut, Roger & Petrusz, 1981; Douglas & Palkovits, 1982) an area which with the nucleus retroambigualis is suggested to be concerned with the generation of respiratory rhythm (Cohen, 1979; Merrill, 1981). It may be noteworthy that the immunoreactivity of the two peptides has been demonstrated in the nucleus ambiguus, an area in close proximity to the nucleus retroambigualis. Perhaps these peptides are involved as neurotransmitters in the physiological regulation of respiration.

There are at present two major hypotheses concerning the mechanism of the generation of respiratory rhythm in the mammalian central nervous system: (1) genesis by the network interaction of neurones and (2) the existence of pace-maker neurones which possess an intrinsic rhythm generation mechanism. Neither hypothesis has been confirmed (Cohen, 1979; Feldman, 1981). In order to establish the existence of pace-maker neurones in the respiratory centre(s), it may be necessary to isolate these neurones completely from the surrounding neurones and other environmental factors (Mitchell & Berger, 1975; Cohen, 1979). The cell culture technique may be suitable for this purpose, but its direct application is likely to be difficult because of (a) insufficient information on the location of the rhythm-generating system(s) in the brain stem and (b) the possible artifacts inherent to the tissue culture technique. Furthermore the failure to find pace-maker neurones in culture experiments does not necessarily mean that the pace-maker cells do not exist in the respiratory centre(s). In this respect, the isolated brain stem–spinal cord preparation of the new-born rat may be useful in clarifying the mechanisms of generation of respiratory rhythm, since it represents an intermediate stage between the intact animal and the dissociated cultured neurones.

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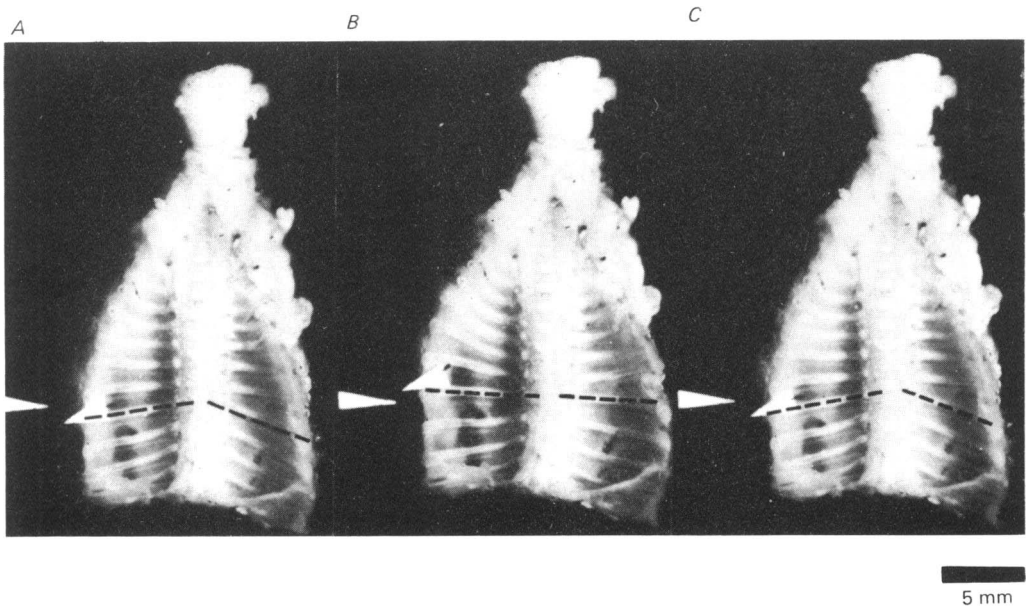
A
B
C
5 mm

EXPLANATION OF PLATE

Isolated brain stem–spinal cord–thorax preparation of a 0-day-old rat, showing spontaneous movement of the thorax. Photographs were taken before (*A*), during (*B*) and after (*C*) the thorax moved spontaneously upward. Photographs *B* and *C* were taken 3 and 5 s respectively after photograph *A* was taken. Two triangular pieces of thin plastic plate were fixed to the preparation and to the bottom of the chamber with thin needles to demonstrate the spontaneous movement of the thorax. Dashed lines along the ninth ribs were drawn on the photograph to show the change in the shape of the thorax. In this preparation, laminectomy was performed on the dorsal side to allow a supply of oxygen to the spinal cord.