REAPPRAISAL FOR PHRENIC MOTOR INNERVATION IN KITTENS BY HRP AND FLUORESCENT DUAL LABELING STUDIES

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Motor innervation of cat diaphragm was studied by retrograde tracer methods using HRP and fluorescent substances of primuline (Pr) and propidium iodide (Pi). In HRP study 20% HRP was injected into the left hemidiaphragm of kittens. After 48 hr animals were perfused with glutaraldehyde and paraformaldehyde. Spinal cord from C₁ to Th₁₃ were resected, cut into 40 μ m serial frozen sections, and provided for TMB reaction. Under the darkfield microscope a cluster of HRP positive neurons was observed in the ventromedial portion of the left anterior horn of the lower cervical (C₅ to C₆) and lower thoracic (Th₉ to Th₁₂) spinal cord, ipsilaterally to the injected side. For the survey of the interrelation of motor innervation between the diaphragm and intercostal muscles, fluorescent dual labeling method was applied. 10% Pr was injected into the left hemidiaphragm, while 3% Pi was injected into the left 8th to 13th intercostal muscles in kittens simultaneously. After 36 hr animals were perfused with 10% formalin. Resected spinal cords were cut into 25 μ m serial frozen sections and observed under the fluorescence microscope. At 360 nm excitation wavelength a small cluster of neurons was illuminated in the specific yellow fluorescence for Pr, while at 550 nm another cluster of neurons was visualized in the specific orange fluorescence for Pi. These two clusters formed a cell group in the ventromedial portion of the anterior horn from 9th to 12th thoracic cords.

Based on these experiments, motor innervation of cat diaphragm can be ascribed not only to the lower cervical but also to the lower thoracic motoneurons, probably via intercostal nerves. The present results might reappraise and consequently support the old hypothesis of "extra-phrenic motor innervation of diaphragm".

The motor innervation of the human and animal diaphragm has been discussed in terms of opposing opinions during the past two centuries. One insists that the phrenic nerve only supplies motor impulses to the diaphragm (2, 11, 16, 20, 21). Another opinion is that besides the phrenic nerve there is one more innervation by intercostal nerves providing efferent components for the diaphragm (5, 9, 19).

Evidence for the innervation by the phrenic nerve has been presented through various histological observations in autopsied or surgical materials, and by electrophysiological studies in experimental animals. Webber *et al.* in 1979 (29), by

intradiaphragmatic injection of horseradish peroxidase (HRP) in cats demonstrated that cat phrenic motoneurons formed a cluster in the most ventral portion of the ventral horn of the lower cervical cord. Their observation was, however, limited only to the cervical cord, giving no comment on the other spinal cord segments, while, there have been a few reports in recent literature supporting the "extra-phrenic motor innervation of the diaphragm" (30).

Recently, a double labeling method using fluorescent substances was established by Kuypers *et al.* (13). By this method, groups of parent cells can be differentially labeled with two fluorescent tracers which are injected into different sites.

Our first attempt was to examine whether the extra-phrenic sources take part in motor innervation of the diaphragm by HRP method in kittens. The result disclosed that HRP positive neurons were still found in the lower thoracic cord, as well as in the lower cervical cord. According to this result, we extended the experiment by the fluorescent labeling method to analyse the interrelation between the diaphragm and intercostal muscles using primuline (Pr) in combination with propidium iodide (Pi) in kittens, as we previously demonstrated the neuronal localization of Onufrowicz's Group-X in rabbits (18).

The present results revealed that the motoneurons in the anterior horn of lower thoracic cords, as well as the lower cervical cord, have a share in innervating motor function of the cat diaphragm, probably via intercostal nerves.

MATERIALS AND METHODS

(I) *HRP method:*

Four kittens (150–350 gr) were anesthetized with pentobarbital sodium. The left thorax of each animal was opened between the 9th and the 10th rib under the positive pressure ventilation via endotracheal catheter. A 100 μ l of 20% HRP (Toyobo, Grade 1-C, RZ 3.4) in Ringer's solution was injected into the left diaphragm of each animal. After a 48 hr's survival period, animals were perfused with fixative solution of 1.25% glutaraldehyde, 1.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Animals were re-perfused with 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose. Spinal cords from C₁ to Th₁₃ were removed and stored overnight in the perfusate at 4°C. The cervical and thoracic cords were then separated into 23 segmental blocks. Each block was cut into 40 μ m serial frozen sections on a freezing microtome. Every 5 serial sections were affixed on the slide glass and processed for tetramethyl benzidine (TMB) reaction after Mesulam (17). Preparations were mounted with balsam and surveyed under the darkfield microscope.

(II) *Fluorescent dual labeling method:*

Four kittens (150–350 gr) were anesthetized in the same manner as HRP method. The left thorax was opened between the 7th and the 8th rib. In two kittens 50 μ l of 20% Pr in physiological saline was injected into the whole area of the left diaphragm, while in the other two kittens Pr was injected exclusively into the central part of the left diaphragm. After Pr injection, the thorax was closed by suture. In each animal, 50 μ l of 3% Pi in distilled water was injected into the left external intercostal muscles from the 8th to the 13th rib. After a 36 hr's survival

period, animals were perfused with 10% formalin in physiological saline. Spinal cords from C₁ to Th₁₃ were removed, cut into 25 μ m serial frozen section. Specimens were affixed on the slide glass, mounted with glycerin, and observed under the Olympus refracted fluorescence microscope. To observe the specific fluorescence of either Pr and/or Pi, the excitation wavelength was arranged at 360 nm and 550 nm, respectively.

RESULTS

(I) *Result for HRP method:*

Under the darkfield microscope, neurons labeled with HRP were observed in the anterior horn of two distant cord sections. They were the lower cervical cord (C) ranging from C₅ to C₆, and the lower thoracic cord (Th) from Th₉ to Th₁₂ (Fig. 1a, b). The neurons were located in the ventromedial portion of the left anterior horn and, assembled closely together, formed a cell group. The number of these neurons comprising the cell group varied from one to ten, and was most densely counted in the preparations of middle C₆ and from lower Th₁₀ to upper Th₁₂ (Fig. 2a, b). HRP positive neurons were not observed in the preparations of the serial cord sections from C₇ to Th₈. There was no HRP labeling in the anterior horn of the contralateral side of the injection.

(II) *Result for fluorescent dual labeling method:*

At 360 nm excitation wavelength, neurons labeled with Pr which was injected into the diaphragm were visualized in yellow in the anterior horn from upper C₅ to lower C₆ in all animals. In two kittens in which Pr was injected into the whole area of the left diaphragm, Pr-positive neurons were observed not only in the lower cervical cord but also in the lower thoracic cord from Th₉ to Th₁₂ (Fig. 3a). In the other two kittens in which Pr was exclusively injected into the central part of the diaphragm, Pr-positive neurons were not noticed in the lower thoracic cord. At 360 nm, not only the specific yellow fluorescence of Pr but also that of Pi in orange could be observed simultaneously as shown in the Fig. 3a. It is for that reason that there is an overlap zone in the specific wavelength of Pr and Pi. The specific orange fluorescence of Pi can be visualized most clearly at 550 nm.

At 550 nm, neurons labeled with Pi which was injected into the left external intercostal muscles between the 9th to the 13th rib were visualized in orange in the left anterior horn of the lower thoracic cord from Th₉ to lower Th₁₂ in all animals (Fig. 3b).

Neurons labeled either with Pr which was injected into the diaphragm, or with Pi which was injected into the lower intercostal muscles, were located in the ventromedial portion of the anterior horn. The cluster of Pi-labeled neurons tended to be distributed more internally compared to that of Pr-labeled ones. Of these two clusters of neurons, a cell group was constructed in the ventromedial part of the anterior horn.

FIG. 1. Photomicrographs of phrenic motoneurons labeled with HRP following left diaphragmatic injection. Ventromedial portion of the left anterior horn of the upper C₉ (Fig. 1a) and the lower Th₁₁ (Fig. 1b). Darkfield. $\times 200$

FIG. 2a. Cross sectional maps of the cell groups of cat spinal cords; visualized by HRP study. Each map represents the projection of HRP labeled phrenic motoneurons onto the transvers plane (dots) for respective cord segments.

2b. Semidiagrammatic representation of the location of HRP labeled neurons in the left anterior horn as indicated by blackened triangles.

DISCUSSION

The present experiment by HRP and fluorescent dual labeling methods in kittens is supposed to demonstrate that the motor function of cat diaphragm can be ascribed to the cluster of motoneurons in the ventromedial portion of the ipsilateral anterior horn of the lower thoracic cords, as well as the lower cervical cords. The area of the cell group in the lower cervical cords is regarded as the phrenic nucleus (29), while that in the lower thoracic cords corresponds to the intercostal nerve nucleus. Based on these findings, it is suggested that a possible role of intercostal nerve is also responsible for the cat phrenic motor innervation, at least for the peripheral portion of the diaphragm.

The opinion of "extra-phrenic motor innervation" has been discussed on occa-

FIG. 3. Fluorescent photomicrographs of motoneurons in the left anterior horn following simultaneous injections of Pr into the left diaphragm and Pi into the left external intercostal muscles from 8th to 13th rib.

3a. Note the neurons illuminating in yellow ($\uparrow$), the specific fluorescence of Pr. At 360 nm. $\times 200$

3b. Another cluster of neurons externally to the Pr-labeled ones illuminating in orange ($\downarrow$), the specific fluorescence of Pi. At 550 nm. $\times 200$

sion for the last one hundred years. Felix (9) in 1922 by histological examination of the serial sections of a human fetus spinal cord, stated that the 12th intercostal nerve supplies the muscle fibres from the same rib and that the crural part is innervated by the phrenic nerves and sympathetic branches from the coeliac plexus.

Schlaepfer (24) in 1926, two years after the transection of the left phrenic nerve in dogs, found that the left half of the diaphragm was completely replaced by a fibrous tissue. He concluded that the phrenic nerve is the only motor nerve for the diaphragm. Similar opinion was reported by Lemon (16). This conclusion was supported by Kiss and Ballon (11). They performed detailed anatomical and histological examinations in human materials of infants and adults, and found the branches ran from every intercostal nerve to the points of costal origin of the diaphragm. By their microscopic observation of these fine branches with osmium staining, they found that about half of the fibers were of the thin type, while the other half had thick medullated sheaths. They did not determine whether these fine fibers were sensory or motor. In conclusion, however, they clearly stated that there is no agreement with the possibility of motor innervation of the diaphragm by intercostal nerves.

Following a thirty year interval of silence in this current of discussion, Last (15) in 1954 described in his textbook that the diaphragmatic branches of intercostal nerves may contain somatic efferent components, and that "the crura, derivatives of the prevertebral rectus, are supplied by the lower intercostal nerves, not by the phrenic." To this description, Botha (3) investigating anatomically the human autopsy materials as well as rats, rabbits, cats and dogs, noticed the fine filaments from the intercostal nerves entering the peripheral diaphragmatic muscle. Regarding them not as the motor nerve, he concluded that the phrenic nerve is the sole motor innervator of the diaphragm.

All the opinions hence discussed came from the observations obtained via indirect approaches. No studies have ever been reported on the "extra-phrenic motor nerve supply of the diaphragm" by direct experimental methods using tracers. Our present results, directly showing the "extra-phrenic motor innervation of cat diaphragm in the lower thoracic cords" seemed to support the hypothesis of the school represented by Felix.

The origin of the phrenic nerve has long been suspected to be located in an area of the anterior horn from the middle to the lower cervical cord. As to its exact localization in the anterior horn there was a diversity of opinions expressed by many workers (4, 6, 8, 12, 22, 27). Keswani and Hollinshead (10) in 1956 constructed a detailed map of the human phrenic nucleus. They studied the cervical cord of men with a previous history of unilateral phrenicotomy by observing the chromatolytic changes of the cresyl violet staining specimens. According to their observation, the human phrenic nucleus was found to occupy the most medial portion of the ventromedial cell groups in the ventral gray horn of the 3rd, 4th and 5th cervical segments. Warwick and Mitchell (28) studied the distribution of motor nerve cells in monkeys by phrenicotomy at varying levels in the neck and thorax to note the acute retrograde degeneration. They showed the presence of the phrenic nucleus as a well-defined group at an intermediate position in the most ventral part of the ventral grey column from the lowest part of the 3rd to the upper third of the 6th cervical segment. After the report by Warwick and Mitchell, similar experimental

studies have been heralded in laboratory animals, such as cats (25) and rabbits (26). Other experimental studies have also been reported by means of electromyography (1, 23) to examine the electrical activity of the phrenic muscle after unilateral phrenicotomy. With these clinicopathological and experimental studies the origin of the phrenic nerve in mammals has been ascribed more evidently to the ventromedial portion of the anterior horn in the middle or the lower cervical cord. Webber *et al.* (29) in 1979 injected HRP into the hemidiaphragm of adult cats to observe the cluster of motoneurons labeled with HRP in the most ventral portion of the anterior horn from 5th to 6th cervical cord. By their HRP study, the phrenic nucleus maps might be directly and exactly demonstrated. Kuzuhara and Chou (14), with a similar method in rats, came to the similar conclusion. However, these two studies by the HRP method described only findings confined to the cervical cord.

So far as the phrenic nucleus localization in the cervical cord is concerned, our present result was well compatible with the previous reports. Our further investigation extended to the thoracic cord showed the presence of another nucleus of the phrenic motor innervation in the lower thoracic cord. Moreover, by the fluorescent dual labeling method, this nucleus in the lower thoracic cord was shown to share the same area with intercostal nucleus. These findings might suggest that the nerves innervating intercostal muscle and the diaphragm, at least its peripheral portion, may originate from a common motor cell group of the anterior horn in the lower thoracic spinal cord.

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