Activation of thalamic ventroposterolateral neurons by phrenic nerve afferents in cats and rats

WEIRONG ZHANG AND PAUL W. DAVENPORT
Department of Physiological Sciences, University of Florida, Gainesville, Florida 32610
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Zhang, Weirong, and Paul W. Davenport. Activation of thalamic ventroposterolateral neurons by phrenic nerve afferents in cats and rats. J Appl Physiol 94: 220–226, 2003.—It has been demonstrated that phrenic nerve afferents project to somatosensory cortex, yet the sensory pathways are still poorly understood. This study investigated the neural responses in the thalamic ventroposterolateral (VPL) nucleus after phrenic afferent stimulation in cats and rats. Activation of VPL neurons was observed after electrical stimulation of the contralateral phrenic nerve. Direct mechanical stimulation of the diaphragm also elicited increased activity in the same VPL neurons that were activated by electrical stimulation of the phrenic nerve. Some VPL neurons responded to both phrenic afferent stimulation and shoulder probing. In rats, VPL neurons activated by inspiratory occlusion would activate these phrenic afferents and also respond to both phrenic afferent stimulation and shoulder probing. Inspiratory occlusion in cats and rats. Inspiratory occlusion can elicit cortical evoked potentials (CEPs) in the somatosensory cortex of cats (11). The majority of CEPs were localized in the vicinity of the postcentral dimple, in the 3a/3b border zone (11, 15, 31), with the use of the cytoarchitectural classification of Hassler and Muhs-Clemment (18). However, the suprapontine projection pathways for phrenic afferents to somatosensory cortex remain unknown.

Retrograde fluorescent tracers injected at the SI cortical sites of primary CEPs elicited by phrenic nerve stimulation labeled cells in the oralis nucleus of the VP complex (VPO) and also the VPL nucleus of the thalamus (31). Thus the phrenic afferent signals could project to SI via the thalamus VPL nucleus. It was hypothesized that electrical stimulation of phrenic afferents will activate neurons in the contralateral thalamic VPL nucleus. If neural responses were elicited with electrical stimulus, the VPL neurons were then tested with mechanical stimulation of the diaphragm and shoulder. Inspiratory occlusion can elicit CEPs in the somatosensory cortex (9). Inspiratory occlusion interrupts the effort of inspiratory muscles and activates muscle mechanoreceptors. Whether phrenic afferents are involved in this process has not been investigated. It was then further hypothesized that inspiratory occlusion would activate these phrenic afferents and also VPL neurons that responded to the direct stimulation on phrenic afferents.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Preparation. The cats (n = 7, weight = 2.4–4.1 kg) were anesthetized with inhalation of halothane-oxygen. Gas anesthesia was then replaced with α-chloralose by slow intravenous infusion (50 mg/kg). Supplemental doses of α-chloralose (5 mg/kg) were administrated throughout the course of the experiment.

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experiment to maintain a deep plane of anesthesia. In rats (Long-Evans hooded rats, n = 4, weight = 400–450 g), the anesthesia was induced with urethane (1.4 g/kg ip). Additional urethane (20 mg) was administrated intravenously as necessary. The adequacy of anesthesia was regularly verified by the absence of a withdrawal reflex (in the unparalyzed state) or blood pressure and heart rate responses (during muscular paralysis) to a paw pinch. Tracheotomy was performed. The femoral artery and vein were catheterized. The body temperature was monitored with a rectal probe and maintained between 37 and 39°C with the periodic use of a heating pad. Arterial blood pressure, end-tidal CO₂, and tracheal pressure were continuously monitored on a polygraph. Arterial blood gases were measured periodically and maintained within physiological limits. The cats were paralyzed with pancuronium bromide (0.6 mg/h iv) and connected to a mechanical ventilator. The rats respired spontaneously.

In cats, a large incision was made in the seventh intercostal space that permitted access to the left phrenic nerve. The most distal 2 cm of the phrenic nerve were isolated, cranial to its attachment to the diaphragm, and placed on bipolar stimulating electrodes. In cats, the phrenic nerve was isolated extrathoracically via a dorsal approach, and the phrenic nerve was confirmed by electrical stimulation.

The animal was then placed in a prone position, and the head and spine were mounted in a stereotaxic apparatus (David Kopf Instruments). A stereotaxic atlas for cats (5) or rats (28) was used during craniotomy. The pericruciate and supraocular regions of the cerebral cortex contralateral to the thoracotomy were exposed in cats. In rats, the craniotomy was opened over the somatosensory cortex by using bregma as the initial point of reference. The dura was reflected. The exposed cortical surface was flooded with warm paraffin oil.

Stimulation and recording. Physiological confirmation of phrenic nerve isolation consisted of (1) recording inspiratory burst activity (in the paralyzed cats) and (2) determination that stimulation produced diaphragmatic contraction without activation of brachial musculature (in rats). The phrenic nerve was stimulated with single electrical pulses (0.1-ms pulse width, S48 stimulator; Grass Instruments, Quincy, MA). In cats, initial stimulation began from 0.1 mA. Intensities of 0.2–0.5 mA were generally used in experiments. In rats, it was 0.020 mA or 0.075–0.200 mA, respectively. Occasionally, higher stimulus intensity was used to investigate the response change. In some animals in which the thalamic neurons responded to the electrical stimulation, thalamic neural responses were also recorded after mechanical stimulation of the diaphragm, shoulder, and abdomen. Diaphragm stimulation was performed with a swab to probe the different regions of the thoracic surface of the muscle. The shoulder probing was performed with the cotton tip of a swab to lightly brush the hair. Squeeze of abdominal muscles was performed manually to increase abdominal pressure.

The extracellular recording of neural responses was carried out with tungsten microelectrodes (impedance = 0.5–5 MΩ at 50 Hz) with a microdrive through dorsoventral tracks penetrating the VP thalamus according to the stereotaxic atlas. The electrode was connected to a high-impedance probe connected to an alternating-current preamplifier (P511, Grass Instruments), band-pass filtered at 0.3–3.0 kHz, displayed on an oscilloscope (Tektronix, model 5111), and recorded on magnetic tape (Vetter, model D, A. R. Vetter, Rebersburg, PA) for off-line analysis.

Histological analysis. In all animals in which phrenic stimulation-elicited thalamic neural activity was recorded, the last recording site was marked at the end of the experiment by electrolytic lesions. The animals were then perfused with heparinized saline followed by 4% formalin. The brain was removed and fixed in buffered 4% formalin. The fixed tissue was then cut coronally into 50-μm-thick sections with a freezing microtome. The sections were mounted and stained with hematoxylin and eosin. The stained sections were examined to identify the lesion and the corresponding electrode tract. In cats, the thalamic nuclei in the VP lateral complex were identified on the basis of the features described by Dykes et al. (13) and the maps of Rinovik (29) and Berman and Jones (5). In rats, the atlas from Paxinos and Watson (28) was used.

Data analysis. The recorded neural activity was digitized with a sampling rate of 1 kHz while playing the recorded data into a computer analysis system (model 1401, Cambridge Electronics Design, CED, Cambridge, UK). The electrical stimulation triggered the collection of 50-ms prestimulus and 200-ms poststimulus neural responses. Individual stimulus events were recorded in computer memory. The individual responses were then recalled from memory, and the electrical stimulus was marked as zero time. The onset latency of the neural response was measured as the time from the electrical stimulus to the first action potential. The duration of the neuron responses was measured as the time from the first action potential until the neuron discharge ceased. Multunit responses were recorded in some electrode tracts (Fig. 1) but not included in unit data analysis. In some recording sites, the activity of two to three neurons could be identified and separated by the amplitude and frequency of the action potentials (Fig. 2A).

The response to mechanical stimulation was recorded from magnetic tape and played onto a computer with a sampling rate of 1 kHz (Chart software, version 4.02, AD Instruments, Castle Hill, NSW, Australia). There was no electrical marker of the onset of the mechanical stimulus, so latency and duration of the response could not be determined. Inspiratory occlusion was presented for one to two inspiratory efforts. The presentation of an occlusion was marked manually.

For the measurements of response latency and duration to each VPL neuron, data were expressed as means ± SE. Because the data failed the normality test, the median value was presented.

RESULTS

In cats, electrical stimulation of the contralateral phrenic nerve evoked excitation of neurons throughout the depth of the VPL nucleus of the thalamus (Fig. 1). No spontaneous activities were observed in these neurons. The single-unit responses showed marked variation of latencies and durations. Forty neurons were recorded in the VPL with a median onset latency of 16.8 ± 0.7 ms (range from 9.0 ± 0.3 to 59.0 ± 1.9 ms) and response duration of 15.1 ± 2.6 ms (range from 4.3 ± 0.5 to 50.1 ± 12.9 ms). Examples of these VPL neuron responses from two animals are shown in Fig. 2. The distributions of the onset latencies of individual neurons are summarized in Fig. 3. Twenty-five of 39 VPL neurons have onset latency <20 ms.

Immediately after the neural response to electrical stimulation of the contralateral phrenic nerve was recorded, neural activities of 23 VPL neurons in cats were examined in response to mechanical stimulation of the diaphragm, shoulder, and/or abdomen (Fig. 4). Nineteen thalamic neurons were tested with direct mechanical stimulation of the diaphragm. Increased

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neural discharge was recorded in 13 of 19 VPL neurons after mechanical stimulation of the diaphragm, and 6 of 19 neurons did not respond to diaphragm probing. Eleven neurons were tested by squeezing the abdomen. Five of 11 neurons increased their activities in response to the abdominal squeeze, whereas others did not respond to this mechanical stimulus. Three neurons increased their discharge in response to both diaphragm probing and abdominal squeeze. Four neurons did not respond to either diaphragm probing or abdominal squeeze. Two neurons were stimulated by diaphragm probing but did not respond to the abdominal squeeze. Nine neurons were recorded with shoulder probing. Four neurons increased their discharge in response to shoulder probing but did not respond to probing of the diaphragm. These four neurons also did not respond to squeezing of the abdomen. Three neurons increased their discharge after both diaphragm and shoulder probing. One neuron increased its discharge in response to all three mechanical stimuli (Table 1). To those VPL neurons activated by diaphragm probing, only the costal region could elicit neural responses. Because of the methodology limitation, surface mapping of diaphragm could not be made. However, it was noted that there would be no neural response beyond a certain area.

In rats, seven VPL neurons increased their discharge in response to electrical stimulation of the contralateral phrenic nerve. These neurons were recorded in the VPL. The median onset latency was 14.3 ± 0.6 ms (range from 9.1 ± 0.6 to 15.6 ± 0.9 ms) with response duration of 13.2 ± 1.9 ms (range from 7.1 ± 1.8 to 18.3 ± 2.5 ms). Phrenic-activated neurons did not show spontaneous activity, so these neurons displayed only excitatory responses. Six of seven neurons were also activated by mechanical stimulation (Table 1). One
neuron was activated by direct mechanical probing on the diaphragm. This neuron also responded to the abdominal squeeze. This was the only neuron exposed to direct diaphragm mechanical stimulation. Three of four neurons tested were activated by squeezing the abdomen. Four of seven neurons were tested with probing of the shoulder. All four neurons increased their activity in response to shoulder probing. Inspiratory occlusion increased the discharge of two VPL neurons tested. These occlusion-activated neurons were also activated by electrical stimulation of the contralateral phrenic nerve and abdominal squeeze. One of these neurons also responded to shoulder probing.

**DISCUSSION**

This study provides the first data that the thalamic VPL nucleus receives afferent information from the respiratory system. Neural activities of VPL neurons were examined with both electrical stimulation of the contralateral phrenic nerve and physiological stimulation of diaphragmatic mechanoreceptors. This study then demonstrated that inspiratory occlusion could activate VPL neurons also activated by phrenic afferents. The present study further demonstrated dual-afferent receptive fields that project to the same VPL neurons from both phrenic afferents and mechanoreceptors of the shoulder.

In the present study, VPL neurons responded to both electrical and mechanical stimuli of phrenic afferents in cats and rats. Previous studies on diaphragmatic mechanoreceptors have described a paucity of muscle spindles (group Ia afferents) but a larger number of Golgi tendon organs (group Ib afferents) in cats and rats (4, 7, 12). The phrenic nerve in cats, in addition to its motor function, is rich in afferent axons. Of the myelinated axons in the phrenic nerve, from 10 to 25% were estimated to be afferents. Twelve percent of the myelinated afferent axons were estimated to supply muscle spindles, with a greater percentage arising from tendon organs (12). Functional studies have found about one-third phrenic of afferent innervated muscle spindless and another one-third innervated tendon organs (3). In adult rats, 43% of the phrenic nerve axons are sensory in origin (25). Thus the diaphragm is innervated by proprioceptors that are able to project afferent information about muscle length and tension to the central nervous system similar to other limb muscles (20). Group I muscle primary afferent fibers from forelimb muscles in the cat ascend in a pathway of the dorsal columns: cuneate nuclei, VP thalamus, and SI cortex (1, 2, 22). On the basis of the similar innervation of proprioceptors with muscle spindles and tendon organs in the diaphragm, it was hypothesized that the projection pathways for diaphragm muscle afferents in the central nervous system might share similar ascending pathways (10). An electrophysiological study has confirmed the projections from the group I and II afferent fibers from the phrenic nerve to the external cuneate nucleus via the dorsal columns (27). However, there is no information on the cuneothalamic projections for phrenic afferents.

In the present study, the phrenic nerve was stimulated with single electrical pulses to primarily activate myelinated phrenic afferents. The neural responses in
the contralateral VPL nucleus were then recorded. The neural responses in the VPL were also tested after mechanical stimulation of the diaphragm. Direct mechanical probing of the diaphragm and squeezing the abdomen to stretch the diaphragm were used to mechanically activate diaphragmatic mechanoreceptors. Diaphragm probing is a more direct stimulus. It involves passive stretch of the muscle fibers and direct pressure on the muscle tissue. Abdominal squeeze also stretched the diaphragm, producing a global mechanical stimulation of the diaphragm by pressing the diaphragm into the thoracic cavity. However, cutaneous receptors of the abdominal skin, abdominal muscle afferents, and visceral receptors may also have been activated by the abdominal squeeze. The same thalamic neurons activated by both electrical and mechanical stimulation on phrenic afferents demonstrated that the pathways activated by electrical stimulation are also involved in the physiological conduction of respiratory muscle mechanical information.

The onset latencies of neural responses in the present study ranged from 9.0 ± 0.3 to 59.0 ± 1.9 ms in cats and from 9.1 ± 0.6 to 15.6 ± 0.9 ms in rats. This wide latency distribution could be partly due to the different afferent fibers activated by electrical stimuli. In cats, the nonmyelinated group IV afferents outnumber the myelinated sensory fibers by a 3:1 ratio. The bulk of the phrenic myelinated sensory fibers is the small-diameter group III afferents (19). In rats, the ratio of the nonmyelinated sensory fibers to myelinated fibers is ~4:3 (25). Electrical stimulation parameters were set to primarily activate myelinated fibers. However, the electrical stimulation procedures used in the present study may not differentiate different afferent populations. Activation of nonmyelinated and/or small-diameter myelinated fibers may have occurred. Early study of the SI cortical representation of phrenic afferents found the mean conduction velocities to be 55.9 and 38.5 m/s, respectively, for primary and secondary peaks (11), which were within the range of group II fibers. Different ascending pathways might also contribute to the different response latencies. The phrenic afferent signals might ascend in two pathways: dorsal column-medial lemniscal pathways and the spinothalamic tract (STT). A report of an electrophysiological study demonstrated that the external cuneate nucleus receives afferent input from the diaphragm (27). STT neurons in the cervical spinal cord in primates can be activated by electrical stimulation of the phrenic nerve (6). Those afferent fibers were identified as group II fibers.
and III fibers. Both of these afferent fiber types can respond to mechanical stimuli. STT inputs could project to VPL in cats, and the spike latency and stimulus threshold showed wide fluctuation (32). Thus, whereas the stimulus parameters probably activated myelinated afferents, the variation in thalamus neural latency response to phrenic nerve stimulation may be due to different types of afferent fibers and different neural pathways.

Activation of the somatosensory cortex in cats after electrical stimulation of the contralateral phrenic nerve has been investigated (11). The majority of CEPs were localized in the vicinity of the postcruciate dimple, in the 3a/3b border zone (11, 15, 31). In cats, the body surface representation is topographically organized in 3a and 3b (14). Most CEP recording sites were located between the forelimb and hindlimb representations, i.e., the trunk region (11, 15). Retrograde labeling study (31) indicated that SI cortical neurons activated by electrical phrenic stimulation have thalamocortical projections that originate from the VP thalamic region (VPO and VPL). VPO is the rostral and dorsal portion of the VP in cats. It contains larger, more darkly stained neurons than those found in the VPL, and these cells are also more sparsely distributed than those in the VPL. This means it might be a different structure than the VPL. The VPO nucleus was hypothesized to be an anatomically distinct functional unit receiving a predominant excitatory input from muscle spindles and projecting to area 3a (13). In the present study, the recording sites of neural activities were restricted to the VPL because of the very rich literature about the role of the VPL nucleus relaying proprioceptive afferents, especially group I afferents of forelimb and hindlimb, to the SI cortex (1, 22, 24).

Neurons in this nucleus were activated after the electrical stimulation of the contralateral phrenic nerve. Comparing the thalamic onset latencies with the previous study on the SI representation of phrenic afferents in cats (11), the largest group of VPL neurons has an onset latency that precedes the onset and peak latency of phrenic afferent activated SI cortex. In the present study, 25 neurons have onset latencies <20 ms, including 9 neurons <12 ms. These results indicate that the neurons in VPL are able to function as a relay for phrenic afferent information to the SI cortex. Considering the similarity of afferent pathways between diaphragm and limb muscles, these results support the hypothesis that the VPL plays an important role in the conduction of proprioceptive information from the diaphragm to the somatosensory cortex.

In spontaneously breathing rats, inspiratory occlusion elicited neural responses in VPL neurons that were activated by electrical stimulation of the contralateral phrenic nerve and abdomen squeeze. These results suggest that phrenic afferent activation is involved in the inspiratory occlusion-elicited cortical somatosensory activity, possibly via a thalamic relay. Inspiratory occlusion-elicited somatosensory CEPs could be recorded with epidural electrodes in conscious lambs (9). In humans, inspiratory occlusion has been found to elicit evoked potentials in the somatosensory region of the cerebral cortex (8, 26). The sources for these respiratory-related evoked potentials are not decided yet. Human study of double-lung transplant patients suggested that the vagal and airway afferents were not necessary for eliciting evoked potentials (33). This suggests that the mechanoreceptors in respiratory muscles provide afferent signals that activate the somatosensory cortex. The present study suggested that phrenic afferents could contribute to inspiratory occlusion-elicited CEPs via the VPL thalamus. No respiratory-phase neural activity could be recorded in these VPL neurons, which suggested that these VPL neurons are generally quiescent during spontaneous breathing until they are activated by specific stimulation of respiratory mechanoreceptors. It is possible that there is a threshold mechanism that prevents afferent information from activating the VPL thalamus or higher central nervous structures during normal breathing. The inspiratory occlusion used in this study may apply extra mechanical stimuli on respiratory afferents to open the “gate,” because, generally, phrenic afferents are found to have little influence on control of normal breathing (16). This selective conduction of respiratory afferent information may be involved in voluntary control of breathing and perception of respiratory mechanical information, because the suprapontine control of breathing must require afferent information to adjust motor drive to respiratory muscles.

Shoulder probing elicited responses in VPL neurons that can be activated by electrical stimulation of the phrenic nerve or mechanical stimulation on the diaphragm. The probing method used in this study mainly activates hair and/or cutaneous receptors of the shoulder. The VPL neurons excited by both cutaneous receptors of shoulder and mechanoreceptors of the diaphragm indicate that there is dual-afferent innervation of some of these thalamic neurons. It has been recognized that phrenic nerve irritation produces referred pain to the shoulder. This type of referred pain has been thought to be due to the visceral and somatic afferent input exciting the same spinal neurons, most notably STT neurons (30). The electrical stimulation of the phrenic nerve increased the discharge rate of the STT neurons with proximal limb excitatory somatic fields (6). It was proposed that the activation of dorsal column neurons by phrenic afferents might account for the different referred pain field sizes in diseases of the diaphragm. The present study supports dual sensory inputs to thalamic neurons from the diaphragm and shoulder afferents. In addition, the STT might be one pathway for diaphragmatic afferents to the thalamus.

In summary, the present study recorded neurons in the VPL nucleus activated by electrical stimulation on the contralateral phrenic nerve in both cats and rats. The majority of these VPL neurons also showed increased neural discharge in response to mechanical stimulation of the diaphragm muscle. The inspiratory occlusion was able to activate phrenic afferent activated VPL neurons in spontaneously breathing rats.
Some neurons responded to both phrenic stimulation and shoulder probing, which might be involved in referred pain from the diaphragm to the shoulder. These results support the hypothesis that the thalamic VPL nucleus can function as a relay for the conduction of proprioceptive information from the diaphragm to the somatosensory cortex.

REFERENCES