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DISTRIBUTION OF RESPIRATION-RELATED NEURONAL ACTIVITY IN THE THORACIC SPINAL CORD OF THE NEONATAL RAT: AN OPTICAL IMAGING STUDY

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Abstract—The inspiratory motor outputs are larger in the intercostal muscles positioned at more rostral segments. To obtain further insights into the involvement of the spinal interneurons in the generation of this rostrocaudal gradient, the respiratory-related neuronal activities were optically recorded from various thoracic segments in brainstem–spinal cord preparations from 0- to 2-day-old rats. The preparation was stained with a voltage-sensitive dye, and the optical signals from about 2.5 s before to about 7.7 s after the peak of the C4 inspiratory discharge were obtained. Respiratory-related depolarizing signals were detectable from the ventral surface of all thoracic segments. Since the local blockage of the synaptic transmission in the thoracic spinal cord induced by the low-Ca^{2+} superfusate blocked all respiratory signals, it is likely that these signals came from spinal neurons. Under the-low Ca^{2+} superfusate, ventral root stimulation, inducing antidromic activation of motoneurons, evoked depolarizing optical signals in a restricted middle area between the lateral edge and midline of the spinal cord. These areas were referred to as 'motoneuron areas'. The respiratory signals were observed not only in the motoneuron areas but also in areas medial to the motoneuron areas, where interneurons should exist; these were referred to as 'interneuron areas'. The upper thoracic segments showed significantly larger inspiratory-related signals than the lower thoracic segments in both the motoneuron and interneuron areas. These results suggest that the inspiratory interneurons in the thoracic spinal cord play a role in the generation of the rostrocaudal gradient in the inspiratory intercostal muscle activity.

Key words: spinal cord, respiratory neurons, neonatal rat.

INTRODUCTION

Respiration involves a complex pattern of movements for which numerous motoneurons distributed along the spinal cord need to fire in the proper spatial and temporal sequences. A number of studies involving electrical recordings from intercostal muscles or nerves in anesthetized cats (Kirkwood et al., 1982; Greer and Martin, 1990), decerebrate cats (Le Bars and Duron, 1984), anesthetized dogs (De Troyer and Ninane, 1986; Legrand and De Troyer, 1999), and humans (De Troyer et al., 2003) have shown that the external intercostal muscles, or their nerve filaments, are active during inspiration, and that the inspiratory activities in the rostral interspaces are stronger than those in the caudal interspaces (see De Troyer et al., 2005 for a review). Similarly, the parasternal region of each interchondral internal intercostal muscle (the so-called parasternal intercostals) is active during the inspiratory phase, and muscles in the rostral interspaces showed stronger activities than muscles in the caudal interspaces in the anesthetized dogs and awake humans (Legrand et al., 1996; Gandevia et al., 2006). Since deafferentation of the rib-cage does not affect the rostrocaudal gradient of the inspiratory motor activity in the parasternal intercostals (Legrand et al., 1996), central respiratory networks would appear to organize this basic spatial and temporal pattern for respiration. It has been shown that this rostrocaudal gradient remained in an isolated brainstem–spinal cord rib preparation obtained from neonatal rats (Iizuka, 2004). However, the neuronal mechanisms responsible for this pattern generation remain unknown.

It is well documented that the bulbospinal neuron provides monosynaptic inputs to the intercostal motoneurons (Davies et al., 1985a,b; Duffin and Lipski, 1987). Although Davies et al. (1985b) showed that external intercostal inspiratory activity was directly relevant to the rostrocaudal gradient, there was no evidence that the inspiratory bulbospinal neurons have systematic patterns of connections to different segments. Thus, the rostrocaudal gradient of external intercostal inspiratory activity would not be explained by monosynaptic inputs from the inspiratory bulbospinal neurons.

Extracellular or intracellular recordings have shown that many thoracic interneurons have respiratory activity (Kirkwood et al., 1988, 1993; Schmid et al., 1993; Saywell et al., 2011). The interneurons projected to the thoracic ventral horn were mainly distributed in the contralateral medial ventral horn in the same spinal segment (Schmid et al., 1993). In all of these electrophysiological studies, however, the recordings were obtained from restrictive thoracic segments, and it is impossible to know

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the rostrocaudal distribution of inspiratory interneurons. Optical recording with a voltage-sensitive dye is very useful for performing macroscopic analysis of respiratory neuron network activity in brain slices or block preparations (Onimaru and Homma, 2003, 2007). Oku et al. (2008), using a voltage-sensitive dye, found a new respiratory neuron group that extends from the medullo-spinal junction to the C2 segment. Here, we examined the distribution of the inspiratory-related optical signals in the thoracic spinal cord using an *in vitro* preparation from neonatal rats.

EXPERIMENTAL PROCEDURES

Ethical approval

The present study was approved by the Animal Research Committee of Showa University.

Brainstem spinal cord preparation

Wistar rats ($n = 24$), 0–2 days of age, were deeply anesthetized with isoflurane until their nociceptive reflexes were abolished. Then, the cerebrum was quickly removed by transection at the intercollicular level, and the brainstem and spinal cord were isolated according to methods described previously (Suzue, 1984; Onimaru et al., 1988). The brainstem was rostrally decerebrated between the 6th cranial nerve roots and the lower border of the trapezoid body.

Optical recordings

Detailed procedures for optical measurement are described elsewhere (Onimaru and Homma, 2003). In brief, the brainstem–spinal cord preparation was stained for 30–55 min with 0.05 mg/ml Di-2-ANEPEQ (Molecular Probes, Eugene, OR, USA), a fluorescent voltage-sensitive dye, in a modified Krebs solution (described below). After staining, the preparation was pinned on the silicone rubber plate (about 1-mm thick, 4–6 mm × 20–25 mm) with the ventral surface up, and then moved to a 2.5-ml perfusion chamber, which was mounted on a fluorescence microscope (BX50WIF-2; Olympus Optical, Tokyo, Japan). In nine preparations, the preparation was bent laterally and three to four fine pins were pinned to the midline of the spinal cords to form a U shape to obtain simultaneous recordings from the upper and lower thoracic cord (see Fig. 5). The preparation was superfused continuously at 2–3 ml/min with modified Krebs solution consisting of (in mM): 124 NaCl, 5.0 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 30 glucose, and equilibrated with 95% O_2 and 5% CO_2, pH 7.4, at 25–27 °C. In some experiments, Ca^{2+} was lowered to 0.2 mM and Mg^{2+} was elevated to 5 mM (referred to as low-Ca^{2+} solution) to locally block the chemical synaptic transmission within the thoracic spinal cord (Onimaru et al., 1989; Kawai et al., 2006).

In three experiments, the chamber was split using the plastic plates described below (1-mm thickness) at the level of the C6–C7 segments in order to separate the spinal cord into the rostral and caudal parts. A semicircular 'bite' was removed from one edge in each of two plastic plates. When the plates were arranged vertically one above the other, these two 'bites' combined to make a hole about 2 mm in diameter in the center. One plate was firmly inserted into the silicone rubber floor. A small amount of Vaseline was smeared on each plate around the hole to make a seal between the two compartments of the chamber. The volumes of the rostral and caudal compartments were about 1.3 and 2.2 ml, respectively. Each compartment was continuously superfused with modified Krebs solution.

Inspiratory motoneuron activity, which was used as the trigger signal for optical recordings, was monitored at the 4th cervical (C4) ventral root with a glass capillary suction electrode. This C4 activity is known to synchronize with discharges of phrenic nerves, which are derived from the C4 and C5 ventral roots (Suzue, 1984). The electrical signal was stored through a 0.5-Hz high-pass filter in digital memory together with the optical imaging data.

Neuronal activity in the preparation was detected as a change in the fluorescence of the voltage-sensitive dye by means of an optical recording apparatus (MiCAM02; Brain Vision Inc., Tsukuba, Japan) through a 510- to 550-nm excitation filter, a dichroic mirror, and a 590-nm absorption filter (U-MWIG2 mirror unit; Olympus Optical) with a tungsten–halogen lamp (150 W) as the light source. The CCD-based camera head has a $4.80 \times 6.40\ mm^2$ imaging area consisting of 124×184 pixels, with a maximum time resolution of 3.5 ms. The magnification of the microscope was ×4.0 times (XL Fluor 4×/340, NA 0.28; Olympus Optical), and the final magnification was adjusted to ×1.4 times (or ×2.0 times) so that an area of $3.43 \times 4.57\ mm^2$ (or $2.40 \times 3.20\ mm^2$) was covered by the image sensor. The focus of the microscope objective was set on the surface plane of the preparation. Respiratory-related neuronal activity was recorded with an acquisition time of 20 ms/frame for 10240 ms (512 frames)/trial, including about 2500 ms before the peak of the C4 inspiratory burst. This measurement was repeated 40 times, and the total of these measurements was averaged. In six preparations, antidromic activation of the thoracic motoneurons was recorded with an acquisition time of 10 ms/frame for 200 ms (20 frames)/trial, including 40 ms before the supramaximal electrical stimulation of the thoracic ventral root (15–50 V, 0.2ms duration, 2-s interval) under low-Ca^{2+} solution. This measurement was also repeated 40 times, and the total of these measurements was averaged.

The fluorescence changes were analyzed with the attached software (BV_Ana(PCI)x86 Edition Version 11.01.20; Brain Vision Inc.) and expressed as a ratio (percentage, fractional changes) (i.e., the fluorescence intensity as a percentage of that of the reference image). The differential image was represented by a pseudocolor display in which red corresponded to a fluorescence decrease, meaning membrane depolarization. To represent the time course of the fluorescence change in the region of interest, optical signals were inverted. In all cases in which the respiratory-related activity was recorded, the differential