The distribution of ganglion cells in the posterior cricoarytenoid muscle of the normal adult rat.  
A light and electron microscopic study

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Summary. We employed by light and electron microscopy to examine the innervation of the posterior cricoarytenoid muscle of the adult rat. The laryngeal nerve was found to often bifurcate into two different bundles. One contained large myelinated (motor) nerve fibers, which were located along the frontal (ventral) muscle surface and entered the muscle at its middle portion to form neuromuscular contacts with individual muscle fibers. The other nerve bundle consisting of clustered ganglion cells (20–30 μm in diameter) and their associated nonmyelinated and small-sized myelinated nerve fibers were mainly found on the dorsal side of the muscle and often ran along the peripheral clefts or depressions of the muscle surface. The nerve bundle often extended side branches, which entered the muscle to be distributed among muscle fibers and near arterioles. Some ganglion cells are considered to enter the muscle, accompanied by branched nerves. Intramuscular ganglion cells and their associated nerve fibers examined by electron microscopy were similar in fine structure to perimuscular ganglion cells and their associated nerve fibers. Nerve fibers contained abundant clear synaptic vesicles which were cholinergic in nature, and often formed synapses with both neighboring axons and the cell body of the ganglion cells. These findings suggest that, in the rat posterior cricoarytenoid muscle, perimuscular and intramuscular ganglion cells exist and may be involved in innervating and contracting smooth muscle cells of the arterioles, thus regulating the blood flow or intravascular pressure.

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Introduction

The intrinsic laryngeal muscles, a group of specialized skeletal muscles, are associated with the regulation of the complex function of the larynx. Many light and electron microscopic studies have been performed on muscle fibers (Matzel and Vosteen, 1963; Shagat and Hast, 1974; Malmgren and Gacek, 1981) and normal, denervated, and reinnervated neuromuscular junctions (Rossi and Cortestina, 1964; Abo, 1975; Morales et al., 1980; Rosen et al., 1983; Yoshiiha et al., 1984, 1991; Michael et al., 1985; Gambino et al., 1985; Freije et al., 1987; Nomoto et al., 1991, 1993; Kawakita et al., 1996, 1998; Yamagata et al., 2000) of these muscles in various animals and the human.

On the other hand, it is of particular interest to note that, in addition to the motor innervation, ganglion cells exist in the intrinsic laryngeal muscle of the dog (Sugano, 1929; Lemere, 1932; Hisa et al., 1996) and rat (Neuhuber et al., 1994). Recent light microscopic histochemical and immunocytochemical studies have also revealed that those ganglion cells are NADPH-diaphorase positive or sensory in nature (Neuhuber et al., 1994, Hisa et al., 1996). However, it is still uncertain whether or not intramuscular ganglion cells exist in all intrinsic laryngeal muscles.

During the course of light microscopical studies by toluidine blue-stained semithin-sections of the rat cricothyroid and posterior cricoarytenoid muscles, we observed ganglion cells in and around the posterior cricoarytenoid muscle, while no ganglion cells seemed to exist in the cricothyroid muscle. These observations suggest that in addition to the species difference, the existence of ganglion cells in the intrinsic laryngeal muscles may differ between muscles even in the same animal. From our preliminary survey, the rat posterior cricoarytenoid muscle was deemed suitable for an investigation of the distribution, nature, role and fine structure of ganglion cells and their associated nerve fibers.
Fig. 1. Three light micrographs of the recurrent laryngeal nerve on the lateral side of a posterior cricoarytenoid muscle. 

a. The recurrent nerve contains myelinated nerve fibers and ganglion cells (small asterisks). b. The nerve is divided into two parts consisting of myelinated nerve fibers and aggregated ganglion cells (small asterisks), which form a side protrusion of the nerve. Note the existence of small-sized myelinated nerve fibers (small arrowheads) around ganglion cells. c. In addition to the myelinated nerve bundle (large arrow), a ganglion (large arrowhead) containing a small number (less than 10) of ganglion cells (small asterisks) is found, possibly indicating the bifurcation of the recurrent nerve. Note small-sized myelinated (small arrowheads) and nonmyelinated nerve fibers around ganglion cells. a–c: ×400

Fig. 2. Light micrographs of the frontal side of a posterior cricoarytenoid muscle. a. A myelinated (motor) nerve bundle (large arrow) enters the muscle at its middle portion. b. Nerve fibers form neuromuscular contacts with individual muscle fibers (arrowheads). a, b: ×400

Fig. 3. Light micrographs of ganglion cells in and around the muscle. a. The nerve bundle consisting of ganglion cells (small asterisks) and their associated small myelinated and nonmyelinated nerve fibers (arrows) runs on the dorsal surface of the muscle. ×400. Inset. Two ganglion cells (small asterisks) near the dorsal surface of the muscle. ×400. b. Two ganglion cells (small asterisks) observed on the peripheral cleft or depression of the muscle. ×400. c. A ganglion cell (small asterisk) is found among muscle fibers at the dorsal side of the muscle. ×400. Inset. A ganglion cell (small asterisk) near the bifurcation of arterioles. ×400
Fig. 2 and 3. Legends on the opposite page.
Therefore, the present study employed light and electron microscopy for a detailed examination of the distribution and functional significance of ganglion cells in the posterior cricoarytenoid muscle of the normal adult rat.

Materials and Methods

Three 3-month-old albino (Wistar) rats, weighing 300-400 g, were used in this study. The animals were housed at a constant temperature (24°C) with a 12:12 light-dark cycle, and given food and water ad libitum. The following experiments were conducted in accordance with the regulations of the Laboratory Animal Research Center for Animal Experimentation at the Ehime University School of Medicine.

After the animals were anesthetized via an intra-abdominal injection of 50 mg/kg pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, USA), they were fixed by cardiac perfusion with a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). After the entire larynx was excised, the posterior cricoarytenoid muscle was dissected out under a stereoscopic microscope, cut into two segments at its equator, and further fixed with a fresh fixative for additional 2 h. They were then washed in a buffer and postfixed in 2% buffered osmium tetroxide for 2 h. The specimens were block-stained with 3% uranyl acetate for 2 h and embedded in Epon epoxy resin after dehydration through a graded series of ethanol. Serial semi-thin sections (0.5 μm thick) were cut with a Porter-Blum ultramicrotome for a distance up to about 500 μm, and were stained with 0.5% buffered toluidine blue for light microscopy. All semithin sections were examined with an Olympus BH-2 light microscope.

In some samples containing ganglion cells observed in toluidine blue-stained semithin sections, ultrathin sections were continuously cut with a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Hitachi HU 12A electron microscope.

Results

Light microscopy

Semithin sections stained with toluidine blue revealed that the recurrent laryngeal nerve in the lateral side of the posterior cricoarytenoid muscle contained some oval- or round-shaped ganglion cells (20-30 μm in the largest diameter) in addition to myelinated nerve fibers (Fig. 1a), and then formed a side protrusion by the aggregation of ganglion cells (Fig. 2b). This laryngeal nerve often bifurcated into two different bundles: one contained large myelinated (motor) nerve fibers, and the other was composed of clustered ganglion cells with small-sized myelinated (about 2 μm in diameter) and nonmyelinated nerve fibers (Fig. 1c).

The myelinated (motor) nerve bundle of the laryngeal nerve ran on the frontal (ventral) surface of the muscle and entered the muscle near its middle region (Fig. 2a). This nerve bundle repeatedly ramified into several branches in the muscle, and finally formed neuromuscular contacts with individual muscle fibers (Fig. 2b).

On the other hand, the nerve bundle containing several ganglion cells and nonmyelinated and small-sized myelinated nerve fibers was mainly distributed along the dorsal surface of the muscle towards its middle portion (Fig. 3a and inset), and occasionally contacted the muscle at its peripheral depressions or clefts (Fig. 3b). There were more than 20 of these perimysial ganglion cells in the semithin sections of six muscles of three animals. In addition to the perimysial ganglion cells, ganglion cells were also found in the muscle. Fifteen intramuscular ganglion cells 20-30 μm in diameter were observed in six muscles. They were distributed mainly solitarily among muscle fibers (nine cases) (Fig. 3c), and were often found near the arteriole and the bifurcation of arterioles (three cases) (Fig. 3c inset). Ganglion cells also existed in the intramuscular myelinated nerve bundle (four cases).

Fig. 4. Electron micrographs of a ganglion cell and its associated nerve fibers in and around the muscle. a. The ganglion cell (large asterisk) covered by a single-layered perineurium (P) exists on the clef of the muscle. Several nonmyelinated nerve fibers (arrows) are wrapped by Schwann cells. Note a myelinated nerve fiber (Mf) within the perineurium. × 3,400. Inset. Two varicose swellings of nonmyelinated nerve fibers (small asterisks) around the cell body contain numerous clear synaptic vesicles, and one varicose nerve swelling forms synapses and/or desmosome-like structures with neighboring nerve fibers (small arrowheads). × 17,000. b. A small nerve bundle (Nb) branching from the perimysial nerve bundle enters the muscle through the interstice between two muscle fibers (M). It consists of several nonmyelinated nerve fibers associated with Schwann cells. × 3,400. c. An intramuscular nonmyelinated nerve bundle cut transversely (Nb) is observed between muscle fibers. × 4,100. d. Nonmyelinated nerve fibers associated with Schwann cells (Nb) are found near and around an arteriole (A). × 8,600.
Fig. 5. Electron micrograph of a solitary ganglion cell among muscle fibers. The ganglion cell (large asterisk) is covered by a single layered perineurium (P) and contains numerous rough endoplasmic reticulum and well-developed Golgi apparatus. Note two myelinated nerve fibers (MF). \( \times 6,300 \). **Inset.** Two varicose nerve swellings around the cell body contain numerous clear synaptic vesicles (small asterisks), and one varicose swelling forms a synapse with the cell body (small arrowhead). \( \times 29,000 \)

Fig. 6. Electron micrographs of a ganglion cell and its associated nonmyelinated nerve fibers near the bifurcation of arterioles observed in Figure 3c inset. **a.** Nonmyelinated nerve fibers are observed around the ganglion cell (large asterisk). \( \times 3,500 \). **Inset.** Several nonmyelinated nerve fibers associated with Schwann cells (arrows) within the perineurium are observed along the circumference of the arteriole (A1). \( \times 4,500. **b.** Several nonmyelinated nerve fibers associated with Schwann cells (arrows) are distributed along the circumference of an arteriole (A2). Note that nonmyelinated nerve fibers enwrapped by a Schwann cell (medium-sized asterisk) are observed at a distance from the arteriole (A2). Sm: smooth muscle cell. \( \times 5,300. **Inset.** Higher magnification of nonmyelinated nerve fibers around the arteriole. Note that varicose nerve swellings enwrapped by the Schwann cell contain clear synaptic vesicles, and two of them (small asterisks) partially lack the covering of the Schwann cell and directly face the smooth muscle cell (Sm). \( \times 25,000 \)
Fig. 6. Legend on the opposite page.
Electron microscopy

In the dorsal surface of the muscle, a few ganglion cells and numerous nonmyelinated and occasional small-sized myelinated nerve fibers were covered by a single layered perineurium (Fig. 4a). Varicose swellings of nerve fibers around the ganglion cell body contained a large number of clear and spherical synaptic vesicles approximately 50 nm in diameter and occasional dense-cored vesicles about 100 nm in diameter, and formed synapses and/or desmosome-like structures with neighboring nerve fibers (Fig. 4 inset). The nerve bundle often extended side branches towards the muscle (Fig. 4b). These side branches mainly contained a number of nonmyelinated nerve fibers associated with Schwann cells, and were frequently observed between muscle fibers (Fig. 4c). Nonmyelinated nerve bundles which lacked the perineurium were often distributed near blood vessels, especially near the arterioles (Fig. 4d).

Like the perimuscular ganglion cells, solitary ganglion cells among the muscle fibers were also covered by a single-layered perineurium (Fig. 5). Around the cell body, a number of nonmyelinated nerve fibers occasionally formed synaptic contacts with the ganglion cell body and with each other (Fig. 5 inset). Synaptic vesicles were mainly clear and spherical and were approximately 50 nm in diameter. Ganglion cells near the bifurcation of the arterioles were also similar in fine structure to the perimuscular ganglion cells (Fig. 6a). The nerve bundle containing nonmyelinated nerve fibers associated with Schwann cells extended towards the circumference of arterioles (Fig. 6a inset). Abundant nerve fibers associated with Schwann cells were distributed around arterioles (Fig. 6b), and some of them were closely adjacent to their smooth muscle cells. Varicose swellings of these nerve fibers contained clear synaptic vesicles, and some of them partially lacked the covering of the Schwann cell and directly faced the smooth muscle cells (Fig. 6b inset).

Discussion

The present study revealed that, in the rat posterior cricoarytenoid muscle, a myelinated nerve (motor nerve) enters the muscle from its middle portion and individual nerve fibers form neuromuscular contacts with individual muscle fibers. It also showed that the nerve bundle containing ganglion cells mainly 20–30 μm in diameter and nonmyelinated nerve fibers, possibly originating from parasympathetic nerve fibers via the recurrent laryngeal nerve, is mainly distributed on the dorsal side of the muscle (the opposite side of the motor nerves), and occasionally on the peripheral clefts or depressions of the muscle. Moreover, electron microscopy demonstrated that nonmyelinated nerve fibers around ganglion cells had numerous spherical and clear synaptic vesicles which are considered to be cholinergic in nature. Interestingly, the perimuscular nerve bundle occasionally extended side branches into the muscle and was distributed around blood vessels, especially around the arterioles. These observations suggest that the perimuscular nerve bundle including ganglion cells, are parasympathetic and/or cholinergic in nature, probably innervating smooth muscle cells of arterioles. A similar finding was reported by Yoshida et al. (1993), who stated that in the cat larynx, laryngeal ganglionic neurons including ganglion cells on the dorsal side of the posterior cricoarytenoid muscle appear parasympathetic or cholinergic in nature by light microscopical immunohistochemistry using antisera against several neuropeptides and AChE histochemistry.

In addition to the perimuscular ganglion cells, the present study clarified the presence of intramuscular ganglion cells in the rat posterior cricoarytenoid muscle; there are solitary ganglion cells which are distributed among muscle fibers and near the bifurcation of arterioles. The individual intramuscular ganglion cells and their associated nerve fibers observed by electron microscopy were similar in structure to the perimuscular ganglion cells and their associated nerve fibers. Nonmyelinated nerve fibers often formed synapses with each other and with the cell body of ganglion cells. This may indicate that most of the intramuscular ganglion cells in this study are parasympathetic or cholinergic in nature, and enter the muscle, probably accompanied by branched nerves from its dorsal side. Our findings also suggest that, in addition to these perimuscular ganglion cells, intramuscular ganglion cells and their associated nerve fibers may be involved in innervating the smooth muscle cells of arterioles, thus regulating the blood flow or intravascular pressure.

With respect to the nature of intramuscular ganglion cells, there are some interesting reports that myenteric ganglia in rat esophagus contain AChE positive and small NADPH-diaphorase positive neurons (Neuhuber et al., 1994; Worl et al., 1994). Neuhuber et al. (1994) have also demonstrated that NADPH-diaphorase positive ganglion cells and varicose fibers are observed in the intrinsic laryngeal muscle of the rat, but these ganglion cells are not involved in the co-innervation of motor endplates (motor endings). Recently, Hisa et al. (1996) have demonstrated in the dog intrinsic laryngeal muscle NADPH-diaphorase positive ganglion cells which are bipolar or pseudounipolar in shape; their varicose positive nerve fibers are observed between muscle fibers, and suggest that these intramuscular ganglion cells may be sensory in nature. From these observations, we envision that the rat posterior cricoarytenoid muscle contains two types of ganglion cells: AChE positive...
(cholinergic) and NADPH-diaphorase (sensory) ganglion cells. Further detailed studies using immunoelectron microscopy are needed to identify the sensory ganglionic cells and their functions.

Several interesting findings have been reported on the relationship between nonmyelinated nerve fibers and skeletal muscle fibers. For example, Barker and Saito (1981) morphologically demonstrated in the cat skeletal muscle that nonmyelinated nerve fibers, being probably sympathetic in origin, are closely associated with muscle fibers. Moreover, Bennett et al. (1973) previously reported the formation of synapses with parasympathetic preganglionic fibers of the thoracic vagus nerve during reinnervation of the hemidiaphragm of the adult rabbit. Recently, Nomoto et al. (1991, 1993) have demonstrated in chronically denervated intrinsic laryngeal muscles of the cat that some of aberrant nonmyelinated (adrenergic and cholinergic) nerves are in synaptic contact with the post synaptic membrane of neuromuscular junctions, and suggested that they play an important role in alleviating muscle atrophy.

A light and electron microscopic study which includes a denervation experiment after transection of the recurrent nerve is now in progress in our laboratory to clarify the origin and functional significance of ganglion cells and their associated nerve fibers in the muscle used in this study.

References


