Journal of Chemical Neuroanatomy 43 (2012) 34–42

Contents lists available at SciVerse ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Localization in the vagal ganglia of calcitonin gene-related peptide- and calretinin-immunoreactive neurons that innervate the cervical and the subdiaphragmatic esophagus of the rat

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ARTICLE INFO

Article history:
Received 1 July 2011
Received in revised form 11 October 2011
Accepted 11 October 2011
Available online 25 October 2011

Keywords:
Immunohistochemistry
Retrograde tracing
Jugular ganglion
Nodose ganglion
Vagal sensory neurons

ABSTRACT

We have determined the localization of calcitonin gene-related peptide-immunoreactive (CGRP-ir) and calretinin-ir neurons in the vagal ganglia that innervate the cervical or subdiaphragmatic esophagus. Many CGRP-ir neurons were found exclusively in the jugular ganglion located in the cranial cavity. Calretinin-ir neurons were distributed throughout the vagal ganglia. Injection of Fluorogold into the cervical esophagus resulted in many Fluorogold-labeled neurons in the jugular and nodose ganglia. Injection of Fluorogold into the subdiaphragmatic esophagus resulted in many Fluorogold-labeled neurons, with most in the nodose ganglion. In the case of Fluorogold injection into the cervical esophagus, double-labeling combining immunohistochemistry and retrograde tracing showed that about 40% of the Fluorogold-labeled neurons in the jugular ganglion express CGRP-like immunoreactivity, and about 20% of the Fluorogold-labeled neurons in both the jugular and nodose ganglia express calretinin-like immunoreactivity. In the case of injection into the subdiaphragmatic esophagus, only a few Fluorogold-labeled neurons express CGRP-like immunoreactivity or calretinin-like immunoreactivity in the vagal ganglia. These results indicate that the cervical esophagus receives projections from many CGRP-ir neurons in the jugular ganglion and from calretinin-ir neurons in the jugular and nodose ganglia, while the subdiaphragmatic esophagus receives projections from only a few CGRP-ir and calretinin-ir neurons in the vagal ganglia.

1. Introduction

The rat esophageal wall receives many sensory afferents from the neurons in the vagal ganglia, including the jugular and nodose ganglia (Neuhuber et al., 2006). Our recent Fluorogold retrograde tracing study (Hayakawa et al., 2011) has shown that the cervical vagus nerve contains many sensory nerve fibers that originate from the neurons in both the jugular and nodose ganglia, while the subdiaphragmatic vagus nerve contains nerve fibers that originate exclusively from the neurons in the nodose ganglion. These results suggest that the cervical esophagus and the subdiaphragmatic esophagus receive projections from different sensory neurons in the vagal ganglia. However, it is not clear which ganglion neurons project to the cervical or the subdiaphragmatic esophagus.

Immunohistochemical studies have shown that there are many neurons containing calcitonin gene-related peptide (CGRP) and calcium binding proteins, including calretinin and calbindin, in the vagal ganglia (Helke and Hill, 1988; Ichikawa et al., 1991; Kuramoto and Kuwano, 1995). CGRP-immunoreactive (-ir) neurons are located mostly in the jugular ganglion, but a few are located in the nodose ganglion (Helke and Hill, 1988; Hayakawa et al., 2011). Thus, it is likely that the CGRP-ir neurons in the jugular ganglion project to the cervical esophagus but not to the subdiaphragmatic esophagus. However, it is not clear to what extent the CGRP-ir sensory neurons in the vagal ganglia project to the subdiaphragmatic esophagus.

Many CGRP-ir fibers are found on the wall of the gastrointestinal tract. The CGRP-ir fibers in the esophagus come from the neurons not only in the vagal ganglia but also in the spinal dorsal root ganglia (Kuramoto and Kuwano, 1995; Dutsch et al., 1998; Berthoud and Neuhuber, 2000). CGRP-ir ganglion neurons are thought to send sensory information not only to the spinal cord but also to the brainstem (Gibbins et al., 1985; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985; Hammond and Ruda, 1991). Double-labeling studies combining immunohistochemistry and retrograde tracing have shown that there are CGRP-ir neurons in the vagal ganglia that innervate the cervical esophagus (Green and Dockray, 1987; Dutsch et al., 1998; Wank and Neuhuber, 2001). However, the number and the ratio of double-labeled neurons to tracer-labeled neurons differed among these studies.

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doi:10.1016/j.jchemneu.2011.10.004

The myenteric ganglia in the gastrointestinal tract receive many sensory nerve endings that form intraganglionic laminar endings (IGLEs), which are thought to convey mechanosensory information (Rodrigo et al., 1975; Fox et al., 2000; Phillips and Powley, 2000; Wang and Powley, 2000; Zagorodnyuk and Brookes, 2000). Anterograde tracing studies revealed that the nodose ganglion neurons send fibers and terminals to the IGLEs, the intramuscular arrays and the villus afferents on the esophagus and the stomach wall (Neuhuber, 1987; Wank and Neuhuber, 2001; Powley et al., 2011). The IGLEs are immunoreactive for both calretinin and calbindin (Kuramoto and Kuwano, 1994; Wank and Neuhuber, 2001). Many calretinin-ir and calbindin-ir neurons are located in the jugular and nodose ganglia (Ichikawa et al., 1991). Double-labeling studies combining immunohistochemistry and retrograde tracing have shown that there are many calcium binding protein-ir neurons in the vagal ganglia that innervate the cervical esophagus (Dutsch et al., 1998; Wank and Neuhuber, 2001). The localization in the vagal ganglia of calretinin-ir neurons that project to the cervical and the subdiaphragmatic esophagus is still not clear.

In the present study, we attempted to determine the precise location in the jugular and nodose ganglia of neurons that project to the cervical and the subdiaphragmatic esophagus. Then we determined which ganglion neurons send CGRP-ir fibers or calretinin-ir fibers to the cervical or the subdiaphragmatic esophagus by using double labeling with immunohistochemistry for CGRP or calretinin and the retrograde tracer Fluorogold.

2. Materials and methods

We used 21 male Sprague-Dawley rats weighing 250–300 g. All surgical procedures were carried out with the animals under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The Animal Care and Use Committee of Hyogo College of Medicine approved the procedures.

2.1. Immunohistochemistry for CGRP and calretinin

To determine the distribution of CGRP-ir and calretinin-ir neurons in the vagal ganglia, six rats were perfused with saline followed by 500 ml of 4% paraformaldehyde–15% picric acid in 0.1 M phosphate buffer at pH 7.4 (PB). The occipital and parietal bones were removed, and then the jugular foramen was opened to expose the vagus nerve entering the medulla oblongata. We then removed the left jugular and nodose ganglia together with the medulla oblongata. The samples were immersed in the same fixative for 1 h and then embedded with 10% gelatin. We made frozen cross-sections or horizontal sections at a thickness of 40 μm. The frozen sections were incubated with a polyclonal rabbit anti-CGRP serum (Yanaihara, Fujinomiya, Japan; Y340, 1:3000) or a polyclonal rabbit anti-calretinin serum (Millipore, Temecula, CA; AB5054, 1:4000) for 1 day. The treated sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200) for 5 h, then incubated with Vectastain® ABC reagents (Vector Laboratories) for one day and, finally, reacted with a solution of 0.1% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.1 M Tris–HCl buffer at pH 7.4 for 5 min to produce brown reaction products. Some sections for control experiments were incubated without the primary antibodies, but with the biotinylated goat anti-rabbit IgG. Photomicrographs of CGRP-ir and calretinin-ir neurons were taken from several sections. Long axes of neuronal somata were measured as cell size by using Image J software.

2.2. Immunohistochemistry combined with retrograde tracing

We used 15 adult male Sprague-Dawley rats weighing 250–300 g. To investigate the distribution of CGRP-ir neurons and calretinin-ir neurons in the vagal ganglia innervating the esophagus, the cervical esophagus or the subdiaphragmatic esophagus was injected with 2% Fluorogold (Flurochrome LLC, Denver, CO). The cervical esophagus was exposed at the level of the thyroid gland, and separated from the trachea and the prevertebral muscles. The subdiaphragmatic esophagus was exposed by opening the abdominal wall, and by pushing aside the liver. Using a glass micropipette (tip diameter = 100 μm) affixed to a 10-μl Hamilton syringe, we injected 6 μl of 2% Fluorogold by pressure into the left side of the cervical esophagus, or the left and ventral sides of the subdiaphragmatic esophagus about 1 cm proximal to the stomach just below the diaphragm. Cotton swabs were used during the injections to prevent the spread of the tracer to adjacent structures. The left and ventral sides of the esophagus receive projections mostly from the neurons of the left vagal ganglion. The right and dorsal sides of the esophagus receive projections mostly from the neurons of the right vagal ganglion. Because it is hard to inject Fluorogold into the whole part of the dorsal side of the esophagus, we injected Fluorogold into the left and ventral sides of the esophagus and observed the left vagal ganglion. For control experiments, we poured 10 μl of 2% Fluorogold to the surface of the cervical esophagus and the prevertebral muscles in one animal, and the abdominal cavity in one animal. Three days after the injection, the animals were perfused with saline followed by 500 ml of 4% paraformaldehyde–15% picric acid in PB. The left jugular and nodose ganglia were removed together with the medulla oblongata as described above, embedded with gelatin, and serial frozen horizontal sections were made at a thickness of 40 μm. The sections were incubated with the rabbit anti-CGRP serum (Yanaihara; 1:3000) or the rabbit anti-calretinin serum (Millipore; 1:4000), and then with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA; 1:500). Fluorogold was viewed using a U excitation filter (blue-white), and Cy3 was viewed using a G excitation filter (red). Fluorescence photomicrographs of Fluorogold-labeled and Cy3-labeled immunoreactive neurons were made with an Olympus BX51 microscope, and merged photographs were made with Photoshop® CS5 software. We counted Fluorogold-labeled neurons that included the nucleus in every section of the vagal ganglia of 13 rats. In consecutive sections, we did not count fainter Fluorogold-labeled somata without a nucleus or with a split nucleus to avoid double counting the labeled neurons. In the merged photographs, the color of Fluorogold was converted to green, Cy3 was converted to red, and then the double-labeled neurons appeared as yellow. By comparing these three kinds of photographs, we counted the neurons that were double-labeled with Fluorogold and Cy3 in every section of the vagal ganglia to determine what is the proportion of neurons that contain CGRP or calretinin between those that innervate the cervical or the subdiaphragmatic esophagus. We then calculated the number of double-labeled neurons per Fluorogold-labeled neuron in the jugular and nodose ganglia.

3. Results

3.1. Immunohistochemistry for CGRP and calretinin

The sensory root of vagus nerve extended from the dorsolateral medulla oblongata, ran through the cranial cavity and emerged to the cervical region from the jugular foramen. In the course of the cranial cavity, the vagus nerve includes the jugular ganglion (about 2 mm long), which is located about 1.8 mm ventrolateral to the medulla oblongata, and the nodose ganglion, which is distal to the jugular ganglion along the internal jugular vein at the jugular foramen (Fig. 1A and B). The boundary between the jugular ganglion and the nodose ganglion could be recognized as a slight indentation. Immunohistochemistry for CGRP showed that numerous CGRP-ir neurons are located in the jugular ganglion (2143.3 ± 51.8, $n = 3$, mean ± S.E.M.), and a few CGRP-ir neurons are located in the nodose ganglion (35.7 ± 6.6, $n = 3$) (Fig. 1A). The CGRP-ir neurons in the jugular ganglion were round or oval cells that ranged from medium-sized to large (29.9 ± 0.4 μm in length, $n = 302$) (Fig. 1C). Immunohistochemistry for calretinin showed that many calretinin-ir neurons are located throughout the jugular ganglion (505.7 ± 81.6, $n = 3$) and the nodose ganglion (377.7 ± 67.4, $n = 3$) (Fig. 1B). The calretinin-ir neurons were also round or oval cells that ranged from medium-sized to large (Fig. 1D). The calretinin-ir neurons in the nodose ganglion (32.1 ± 0.5 μm in length, $n = 222$) were slightly larger than those in the jugular ganglion (29.4 ± 0.4 μm in length, $n = 308$). No immunoreactive neurons were found in the vagal ganglia in the sections processed without the primary antibodies.

3.2. Immunohistochemistry for CGRP or calretinin combined with retrograde tracing

When Fluorogold was injected into the cervical esophagus at the level of the thyroid gland, many retrogradely Fluorogold-labeled neurons were found not only in the jugular ganglion but also in the nodose ganglion (Fig. 2A). Double-labeling immunohistochemistry for CGRP and Fluorogold revealed that there were Fluorogold-labeled, Cy3-labeled CGRP-ir, and double-labeled neurons by creating three kinds of fluorescence micrographs by viewing the sections with a U or a G filter, and then merging the two micrographs with Photoshop® CS5 (Fig. 2D–F). In the jugular ganglion, the Fluorogold-labeled neurons overlapped with the