Distribution of calcitonin gene-related peptide nerve fibers in the canine larynx

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Summary. Immunohistochemistry was used to investigate the distribution pattern of calcitonin gene-related peptide (CGRP) nerve fibers in the laryngeal mucosa, glands and intrinsic muscles of the dog. CGRP immunoreactive nerve fibers were found more frequently than substance P immunoreactive nerve fibers in every region of the larynx. In the epithelia, CGRP nerve fibers were mainly found in the epiglottis, arytenoid region and subglottis. Many taste buds were observed in the arytenoid region and were densely innervated by the CGRP nerve fibers. In the lamina propria, the plexus of CGRP nerve fibers was present, with some of these fibers associated with blood vessels. Laryngeal glands were also innervated by a few CGRP nerve fibers. In the intrinsic laryngeal muscles, abundant immunoreactivity was observed and many motor end-plate-like structures were found with CGRP immunoreactivity. These findings strongly suggest that CGRP plays an important role in all of the sensory, motor and autonomic nervous systems of the larynx.

Key words: Calcitonin gene-related peptide – Canine larynx – Immunohistochemistry

Introduction

The innervation of the larynx by several kinds of neuropeptides in addition to the classical neurotransmitters has been shown in previous reports such as ours which demonstrated the distribution of substance P (SP) nerve fibers in the canine larynx [6]. It is well known that calcitonin gene-related peptide (CGRP), one of these neuropeptides, is distributed throughout the central and peripheral nervous systems as a neurotransmitter or a neuromodulator [9, 18]. Although the existence of CGRP nerve fibers in the larynx has also been documented [9], the details concerning its laryngeal innervation are still lacking.

Materials and methods

Five young dogs of both sexes, weighing 1–2.5 kg, were used in this experiment. Under deep anesthesia with i.p. pentobarbital (30 mg/kg), each animal was perfused through the left cardiac ventricle with 0.1 M phosphate-buffered saline (PBS) followed by ice-cold fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.35% glutaraldehyde in 0.1 M phosphate buffer (PB). After perfusion fixation, the larynx was removed and postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB for 1 day. The extirpated larynx was next immersed in 0.1 M PBS containing 20% sucrose for 1 day. It was then sectioned at 20 µm on a freezing microtome. These sections were processed for immunohistochemistry. Free-floating sections were treated with 1% bovine serum for 1 h and incubated with anti-CGRP serum (Amersham, x 5000) for 4 days at 4°C. After perfusion fixation, the larynx was removed and postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS for 1 day. The extirpated larynx was next immersed in 0.1 M PBS containing 20% sucrose for 1 day. It was then sectioned at 20 µm on a freezing microtome. These sections were processed for immunohistochemistry. Free-floating sections were treated with 1% bovine serum for 1 h and incubated with anti-CGRP serum (Amersham, x 5000) for 4 days at 4°C. Sections were then incubated with biotinylated anti-rabbit IgG (x 1000) for 1 day and with avidin-biotin complex (x 1000) for 1 day. Sections were washed in 50 mM TRIS buffer. This was followed by incubation with 0.01% 3,3' diaminobenzidine and 0.3% nickel ammonium sulfate. The sections were next mounted on chrome-coated glass slides, air-dried and washed with tap water. Finally, tissues were dehydrated in a graded series of ethanol, cleaned in xylene and covered with Entellan (Merck, Darmstadt, FRG).

Results

CGRP immunoreactive nerve fibers were found in every region of the larynx. In the epithelia of the laryngeal mu-
CGRP nerve fibers were especially found in the epiglottis, posterior glottis and subglottis (Table 1). In the glottis, abundant CGRP nerve fibers were observed in the posterior glottic epithelium, although there were very few in the anterior glottis. Most of these fibers with varicosities reached the surface of the epithelium and appeared to be free-ended (Fig. 1A). In the epithelium of the laryngeal surface of the epiglottis and the posterior glottis, taste buds were observed and were densely innervated by CGRP nerve fibers (Fig. 1B). In the lamina propria, the plexus of CGRP nerve fibers was commonly found and some of the CGRP nerve fibers were clearly associated with blood vessels (Fig. 1C). No CGRP immunoreactive epithelial cells were found.

Many CGRP nerve fibers were found in the region of laryngeal glands (Fig. 1D). Some of these fibers appeared to terminate in glandular cells, while most fibers were observed to lie parallel to blood vessels.

<table>
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<tr>
<th>Region</th>
<th>Epiglottis</th>
<th>False cord</th>
<th>Glottis Anterior</th>
<th>Glottis Posterior</th>
<th>Subglottis</th>
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<td>CGRP</td>
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Fig. 1. A Subglottic epithelium: free-ended nerve fibers with varicosities reach the surface of the epithelium (→), × 440. B In the epithelium of the arytenoid region, many taste buds are observed and densely innervated by the calcitonin gene-related peptide (CGRP) nerve fibers (→), × 440. C In the lamina propria of the laryngeal epithelium, the plexus of CGRP nerve fibers is commonly found and some of these fibers (→) are clearly associated with blood vessels, × 440. D Many CGRP nerve fibers are observed in the glandular region and some of these fibers (→) appear to terminate in glandular cells, × 220

Fig. 2. Thyroarytenoid muscle having abundant CGRP immunoreactivity in the middle of the muscle (→), × 44. Inset: Under high magnification, motor end-plate-like structures with CGRP immunoreactivity can be observed, × 440.