A Dense Plexus of CGRP-Immunoreactive Nerve Fibers in Portions of the Major Salivary Excretory Ducts Close to Their Opening into the Oral Cavity of Rats

Miyuki YAMAMOTO and Hisatake KONDO
Department of Anatomy, Kanazawa University School of Medicine, Kanazawa, Japan

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Summary. A dense plexus of CGRP-immunoreactive nerve fibers was found subjacent to and within the epithelium of the excretory ducts of the parotid, submandibular and sublingual glands in the rat, in close proximity to the opening into the oral cavity. The immunoreactive nerve fibers disappeared after a neonatal administration of capsaicin. In immuno-electron microscopy, the immunoreactive nerve fibers were characterized by abundant small clear vesicles mixed with some large granular vesicles, but not by any particular abundance of small mitochondria. Intraepithelial immunoreactive fibers were directly apposed to adjacent epithelial cells without any membrane specializations. They did not exceed the subapical junctional complex of the epithelial cells.

Calcitonin-gene related peptide (CGRP) is a peptide composed of 37 amino acids; its immunoreactivity has been demonstrated to occur widely in the central and peripheral nervous system. By means of immunohistochemistry combined with retrograde tracing analysis or pretreatment with capsaicin, a neurotoxin specific for the primary sensory neuron of Aδ and C types, it has been shown that CGRP-immunoreactive nerves in the periphery, except for those related to the skeletal muscle cells, represent peripheral processes of the primary sensory neurons (ROSENFELD et al., 1983; RODRIGO et al., 1983a, b; CADIEUX et al., 1986; SU et al., 1986; GREEN and DOCKRAY, 1987; KUWAYAMA et al., 1987; MOLANDER et al., 1987; SPRINGALL et al., 1987).

In the present study the localization of CGRP-immunoreactive nerve fibers was examined in the parotid, submandibular and sublingual glands and their excretory ducts in the rat. A dense plexus of the immunoreactive fibers was revealed as occurring in portions of the extraglandular excretory ducts close to their opening into the oral cavity. The fine structure of the intraepithelial CGRP-immunoreactive sensory nerve fibers was also described in detail.

MATERIALS AND METHODS

Under Nembutal anesthesia (30 mg/kg body weight), young adult rats weighing 150–200 g were perfused through the heart: first with 200 ml physiological saline, followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. In addition, several newborn rats received only one injection of capsaicin (50 mg/kg, i.p.) in emulsion (Tween 80, saline, absolute alcohol, 1:8:1 by volume). The other newborn rats received equal volumes of capsaicin-free emulsion only and were used as the control. The treated rats and the controls were killed 4 weeks after the operations. The parotid, submandibular and sublingual glands, together with their excretory ducts including portions of the opening into the oral cavity, were removed and immersed in the same fixative for 4 h. After a rinse in the phosphate buffer, the tissue blocks were immersed overnight in 30% buffered sucrose at 4°C. They were sectioned on a cryostat at a thickness of 15–20 μm and incubated with the antisem against CGRP (RPN 1842, Amersham International PLC, Buckinghamshire, U. K.) at a dilution of 1:2,000 for 12 h at room temperature. The antigen-antibody reaction sites were made visible using the peroxidase-antiperoxidase (PAP) method by STERNBERGER

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Fig. 1. A section of the parotid gland immunostained for CGRP. Sparse CGRP-immunoreactive nerve fibers (arrow) are seen along the small blood vessel (V). D interlobular excretory duct. ×290

Figs. 2 and 3. Sections of the submandibular duct (M), the sublingual duct (L), and the parotid duct (P) running in the submucous connective tissue wall of the oral cavity (C). Dense plexuses of CGRP-immunoreactive nerve fibers are found subjacent to and within the epithelium of the excretory ducts. Note the much smaller number of CGRP-immunoreactive nerve fibers in the epithelium and the subepithelial connective tissue of the oral mucosa (O). ×145

Fig. 4. An portion of the parotid duct (P) opening into the oral cavity (C). The CGRP-immunoreactive nerve plexus becomes less dense immediately before the opening. ×145

Fig. 5. A higher magnification of the submandibular duct (M) in close proximity to its opening into the oral cavity. Within the epithelium, CGRP-immunoreactive nerve fibers take a tortuous course; some of them (arrows) appear to reach the lumen of the duct. ×425

Fig. 6. A section of the parotid duct (P) in close proximity to its opening into oral cavity; from a 4-week-old rat neonatally treated with capsaicin. Almost all CGRP-immunoreactive nerve fibers are absent in the epithelium and the subepithelial connective tissue of the duct. ×290
For immuno-electron microscopy, the sections immunostained by the PAP method were postfixed in 1% OsO₄ in cacodylate buffer, pH 7.4 for 10 min and stained in 1% uranyl acetate for 10 min. They were briefly dehydrated in an alcohol series and embedded in Epon 812.

For the specificity control, an absorption test was performed using diluted antiserum pretreated with synthetic CGRP at a concentration of 10 μg/ml for 24 h at 4°C.

RESULTS

CGRP-immunoreactive nerve fibers were rarely found around the excretory ducts within the parotid, submandibular and sublingual glands, although a few of them were seen in association with small blood vessels (Fig. 1). The immunoreactive nerve fibers remained small in number around extraglandular portions of the excretory ducts traveling on the surface of the masseter and digastricus muscles. As the excretory ducts of the parotid, submandibular and sublingual glands proceeded into the submucous connective tissue wall of the oral cavity, CGRP-immunoreactive nerve fibers in association with these ducts increased in number progressively (Figs. 2-4). The immunoreactive nerve fibers appeared in the form of punctate structures or thin processes with varicosities; they were located subjacent to and within the epithelium of the excretory ducts, which was composed of two to four cell layers. The immunoreactive nerve fibers within the epithelium took a tortuous course, with some of them seemingly reaching the surface of the epithelium (Fig. 5). The subepithelial and intraepithelial plexuses of CGRP-immunoreactive nerve fibers were less dense shortly before the excretory ducts opened to the oral cavity (Fig. 4).

In 4-week-old rats which had received a neonatal injection of capsaicin, CGRP-immunoreactive nerve fibers were almost completely absent underneath and

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**Fig. 7.** Immunoelectron micrograph of the epithelium of the parotid duct in close proximity to its opening into the oral cavity. Many electron dense CGRP-immunoreactive nerve fibers (arrows) occur among the epithelial cells. **LP** lumen of the parotid duct. ×8,100
within the epithelium of the whole trajectory of the excretory ducts of the three major salivary glands including portions close to their opening to the oral cavity. A few immunoreactive fibers, however, were still seen in deep portions of the subepithelial connective tissue (Fig. 6).

In electron microscopy, CGRP-immunoreactive nerve fibers were identified as electron-dense neuronal profiles, 0.2-1.3 μm in diameter (Fig. 7). Together with non-immunoreactive neuronal profiles, these fibers were surrounded by Schwann cells in the subepithelial connective tissue. No myelinated nerve fibers were immunoreactive for CGRP (Fig. 8). The immunoreactive fibers were partially denuded of Schwann cell sheaths and directly exposed to the subepithelial connective tissue spaces with only an external lamina. The immunoreactive nerve fibers lost their Schwann cell sheath and the external lamina when they penetrated into the epithelium, and eventually were directly surrounded by the epithelial cells. Smaller neuronal profiles immunoreactive for CGRP contained neurofilaments and neurotubules and several vesicular elements, while larger neuronal profiles immunoreactive for CGRP contained numerous small clear vesicles, 40-60 nm in diameter, mixed with several large granular vesicles, 90-120 nm in diameter and a few mitochondria (Figs. 9, 10). The immunoreactive material was localized in the core of the granular vesicles and also recognized diffusely in the axoplasm; hence the interior of the neuronal profiles was often obscured. The interior of the small clear vesicles and mitochondria was free of the immunoreaction. No immunoreactive neuronal profiles were located beyond the junctional complex formed at the apical portion of the surface epithelial cell of the excretory ducts (Fig. 10). Neither any membrane specializations nor accumulations of vesicles were formed at the apposition sites between the immunoreactive neuronal profiles and the epithelial cells.

After incubation with the control serum, none of the immunoreactive nerve fibers were recognized in any portions of the salivary glands and their excretory ducts.
DISCUSSION

The present study in rats revealed for the first time an extremely dense, subepithelial and intraepithelial plexus of CGRP-immunoreactive nerve fibers in portions of the excretory ducts of the three major salivary glands close to their opening into the oral cavity, in contrast to their actual absence in other portions of the excretory ducts. Since a few CGRP-immunoreactive nerve fibers were sparsely distributed along the whole trajectory of the excretory ducts of the lacrimal and nasal glands (unpublished), such a circumscribed appearance of the dense plexus of CGRP-immunoreactive nerve fibers seems to be characteristic of the salivary glands. This finding suggests that the distal portion of the excretory ducts plays some important roles in the final stage of saliva secretion.

The sensory nature of the peripheral CGRP-immunoreactive nerves has been well documented (ROSENFELD et al., 1983; GIBSON et al., 1984; RODRIGO et al., 1985a, b; CADIEUX et al., 1986; SU et al., 1986; GREEN and DOCKRAY, 1987; KUWAYAMA et al., 1987; MOLANDER et al., 1987; SPRINGALL et al., 1987). This was also confirmed by the present finding that CGRP-immunoreactive nerves at the loci in question of the excretory ducts decreased in number remarkably after the neonatal treatment with capsaicin. It is known that CGRP-immunoreactive sensory nerves participate in nociception (ROSENFELD et al., 1983; GIBSON et al., 1984). The nociception from the circumscribed loci in close proximity to the opening of the salivary duct into the oral cavity, which could occur under some pathological conditions, may help the accomplishment of a normal orthodromic flow of saliva.

On the other hand, recent studies have shown that CGRP is released from cultured trigeminal sensory neurons and that CGRP is a potent vasodilator (FISCHER et al., 1983; BRAIN et al., 1984). These previos findings suggest the possibility that the CGRP-immunoreactive nerve fibers might exert, in addition to the sensation, some efferent influence on the epithelial cells of the excretory ducts. Whatever the real function of the CGRP-immunoreactive nerve fiber may be, more attention should be given to the distal excretory ducts of the major salivary glands when considering the mechanism of saliva secretion.

It has been often documented that, in comparison with efferent nerve ending, the sensory nerve endings are in general characterized by abundant small mitochondria and relatively smaller numbers and more varying sizes of small clear vesicles. Such observations are based on the findings for encapsulate nerve endings such as those in the Pacinian corpuscles (PEASE and WILLIAM, 1957; POLÁČEK and MAZANEC, 1966; NISHI et al., 1969; SPENCER and SCHAUERBACH, 1973). However, the ultrastructural characteristics of the free nerve endings of sensory nature have been poorly understood, mainly because definitive criteria for the identification of the real free nerve endings has been lacking in conventional electron microscopy. Although there have been several reports describing the ultrastructure of the intraepithelial, presumably free and sensory, nerve endings in various regions of the body (MUNGER, 1965; KADANOFF, 1971a, b; TSUJI, 1971; HALATA, 1972; KING et al., 1974; WALSH and MCLELLAND, 1974; DAS et al., 1978, 1979; ROBLES-CHILLIDA et al., 1981; SILVERMAN et al., 1986), all the findings have been obtained solely from fortuitous ultrathin sections. As a result, they have been fragmental at best.

In contrast, the present study revealed clearly and reliably the ultrastructural features of real free nerve fibers of sensory nature. This was accomplished by correlative observations between light and electron microscopic immunohistochemistry using CGRP-like immunoreactivity, which is regarded as a specific marker of at least one population of the sensory nerves. As a result, the presence of abundant small mitochondria was revealed not to be a characteristic of the free nerve ending, unlike the encapsulated nerve endings. This difference in ultrastructure may represent the difference in the mechanism of sensory transduction between a free nerve ending and an encapsulated one.

REFERENCES


