Immunoelectron Microscopic Observation of Calcitonin Gene-Related Peptide (CGRP)-positive Nerves in the Dental Pulp of Rat Molars*

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Summary. The ultrastructure of nerves containing immunoreactivity for calcitonin gene-related peptide (CGRP) was investigated in the dental pulp of rat molars. The immunoreactivity was recognized predominantly in unmyelinated nerve fibers, and sparsely in a few myelinated fibers. It was localized throughout the axoplasm, as well as in the large cored vesicles. Small clear vesicles and mitochondria were free of the immunoreaction. The CGRP-immunoreactive nerves were frequently observed to terminate, being devoid of Schwann cell investment, in the vicinity of blood vessels in the coronal pulp, suggesting that CGRP may be involved in the regulation of pulpal blood flow. Moreover, CGRP-immunoreactive axon terminals containing numerous small clear vesicles, a few large cored vesicles and mitochondria were recognized in contact with the cell bodies of odontoblasts and their processes in the dentinal tubules. Although specialized synaptic ultrastructures were not recognizable, a functional association of CGRP nerves and odontoblasts was suggested. Thus, CGRP in the dental pulp appears to have multiple functions, including vascular regulation and sensory transduction.

Dental pulp contains numerous nerve fibers, the major population of them originating from the trigeminal ganglion (BYES, 1984; FRANK and NALBANDIAN, 1989). Distribution of the pulpal nerves and location of their terminals have long been investigated by various histological techniques (for reviews, FEARNSHEAD, 1967; GUNJI, 1982; BYERS, 1984; FRANK and NALBANDIAN, 1989).

The morphological investigation of pulpal nerves in the pulpodentinal border zone seems most important for understanding the sensory mechanism of dentinal sensation. In this border zone, previous studies have shown that the pulpal nerves take various courses and that all nerve fibers terminate in the predentin and dentin as free endings, filled with a number of mitochondria (GUNJI, 1982; BYERS, 1984). In addition to the mitochondria-rich nerve terminals, some studies indicated the existence of another type of nerve terminal which contained various kinds of vesicles (FRANK, 1966; ARWILL et al., 1973; CORPRON and AVERY, 1973). However, the nature and detailed morphology of both types of nerve endings has remained to be clarified.

Recent immunohistochemical studies have revealed that nerve fibers in the dental pulp contain several neuropeptides including vasoactive intestinal polypeptide (VIP), substance P, and calcitonin gene-related peptide (CGRP) (UDDMAN et al., 1986; WAKISAKA et al., 1985; SILVERMAN and KRUGER, 1987). Among these neuropeptides, CGRP seems one of the most important because of its dense distribution in the dental pulp (HOSHINO et al., 1987; SILVERMAN and KRUGER, 1987). Accordingly, immunohistochemistry for CGRP has been applied to investigate the neurophysiology of dental pulp, and suggested a possible role of CGRP as a vasoregulator in the dental pulp (UDDMAN et al., 1986; WAKISAKA et al., 1987). Further, dynamic responses of CGRP-immunoreactive nerve fibers to external stimuli including dentinal injury have been demonstrated, suggesting CGRP involvement in tissue repair as well as in sensory and/or vascular regulation (KIMBERLY and BYERS, 1988; KHAYAT et al., 1988; TAYLOR et al., 1988; BYERS et al., 1990). While the

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The distribution of CGRP-immunoreactive nerves in the dental pulp has been demonstrated at the light microscopic level, little information has been obtained regarding their fine structural details.

The present study was therefore undertaken to clarify the ultrastructure of nerve fibers containing CGRP in the dental pulp of rat molars by means of immunohistochemistry for CGRP at the electron microscopic level. It also aims to provide morphological evidence elucidating the function of this neuropeptide in the tooth.

MATERIALS AND METHODS

Five adult Wistar rats, weighing 150–200 g, were used in this study. The animals were fixed with a transcardiac perfusion with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Their upper jaws—including molar teeth—were immediately dissected out and immersed in the fixative without glutaraldehyde for additional 6 h at 4°C. After decalcification with 10% EDTA-2Na (pH 7.4) for 3 weeks, the tissue blocks were immersed in a 30% sucrose solution overnight, and rapidly frozen in liquid nitrogen. Tissue sections, 20 μm in thickness, were made in a cryostat.

For immunohistochemistry for CGRP, free floating sections were processed for the streptavidin-biotin-peroxidase method with a commercially available kit (Histofine SAB-PO kit, Nichirei Co. Ltd., Japan). After treatment with 2.5% normal goat serum, the sections were primarily incubated with an anti-rat CGRP serum raised in a rabbit (Cambridge Res. Biochem., England) and diluted 1 : 3000 plus 2.5% normal goat serum for 48 h at 4°C, followed by consecutive two incubations with biotinylated anti rabbit IgG and peroxidase-conjugated streptavidin, each for 12 h at 4°C. Antigen-antibody reaction sites were visualized by 0.04% diaminobenzidine in 0.06 M Tris-HCl buffer (pH 7.6). Immunostained sections were postfixed in 1% osmium tetroxide reduced with 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, dehydrated through a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were made and examined under a Hitachi H-7000 transmission electron microscope without any electron staining. Some immunostained sections were prepared for conventional light microscopic observation.

The specificity of the immunoreactions was tested using antiserum preincubated with the antigen (10 μg/ml diluted antiserum). The antigen-absorbed antiserum did not stain any cellular elements.

RESULTS

Light microscopic observation of CGRP-immunoreactive nerves

Distribution of CGRP-immunoreactive nerve fibers in the dental pulp of rat molars coincided with that reported in previous literature (HOSHIINO et al., 1987; KIMBERLY and BYERS, 1988; KHAYAT et al., 1988). CGRP-immunoreactive nerve fibers, beaded in profile, entered the dental pulp through the apical foramen. They
ran through the center of the radicular pulp without obvious branching. In the coronal pulp, the fibers began to repeatedly branch, eventually distributing extensively throughout the region (Fig. 1). They were concentrated and intermingled beneath the odontoblast layer to form a subodontoblastic nerve plexus of Raschkow. CGRP-positive nerves with varicosities emerged from the plexus towards the pulpodentinal border. Some of them terminated in the odontoblast layer, whereas others passed through the layer, following a straight course to terminate in the predentin and dentin.

Fig. 2. Electron micrograph of a nerve bundle running in radicular pulp, immunostained by the pre-embedding method for CGRP. The immunoreaction products are localized in the axoplasm of unmyelinated nerve fibers (arrows) in the nerve bundle, while a myelinated fiber (⋆) does not show any CGRP-immunoreactivity. ×16,000

Fig. 3. A nerve bundle in the radicular pulp, including a CGRP-immunoreactive axon terminal (⋆) which is exposed from Schwann cell investment. Immunoreaction products are localized in the cytosolic component of the axoplasm, while mitochondria (Mt) and small clear vesicles are free of them. Note that an axoplasm proximal to the immunoreactive terminal portion (arrowhead) is devoid of the immunoreactivity. ×30,000

Fine structure of CGRP-immunoreactive nerve fibers

Nerve fibers immunoreactive to CGRP antiserum were easily identified under the electron microscope by electron dense precipitates occurring in their axoplasm. The major population of the CGRP-immunoreactive nerve fibers was unmyelinated, while only a few were thinly myelinated (Figs. 2, 5).

The myelinated fibers showing CGRP-immunoreactivity contained only a few small vesicles besides mitochondria and numerous filaments. No obvious
accumulation of the vesicles—including large cored vesicles—was found in this type of myelinated nerve fiber. The immunoreaction products were extensively distributed in the cytosolic compartment of the axoplasm; mitochondria and small clear vesicles were devoid of the immunoreaction.

The unmyelinated fibers showing CGRP-immunoreactivity displayed intra-axonal features identical to those of immunoreactive myelinated fibers, except for the occurrence of numerous small clear vesicles, 40–80 nm in diameter, and several large cored vesicles, 120–150 nm in diameter. Both types of vesicles tended to be concentrated in the varicose swellings of the unmyelinated fibers. The immunoreaction products were confined to the core of large cored vesicles as well as in the axoplasm. Small clear vesicles and mitochondria lacked CGRP-immunoreaction. Thin interswollen portions of the axons also frequently lacked the CGRP-immunoreactivity (Fig. 3). No immunoreaction product was observed in any other cellular elements such as fibroblasts, odontoblasts, or Schwann cells.

In the radicular pulp, nerve bundles contained both myelinated and unmyelinated fibers. While the major population of the unmyelinated nerve fibers showed CGRP-immunoreactivity, the myelinated fibers were mostly lacking in the immunoreactive substance (Fig. 2). The immunoreactive axons were incompletely

**Figs. 4-6.** Electron micrographs of nerve fibers distributed in the subodontoblastic region. **Fig. 4.** Some of unmyelinated nerve fibers composing the subodontoblastic plexus show CGRP-immunoreactivity (arrows). **Fig. 5.** A rare case of a thin myelinated fiber containing the immunoreactivity (arrow). **Fig. 6.** A CGRP-immunoreactive nerve fiber that appears to terminate freely in the vicinity of a blood vessel (BV) without making contact with the vessel wall. Fig. 4: ×19,000, Fig. 5: ×12,000, Fig. 6: ×15,000
Fig. 7. a. A CGRP-immunoreactive nerve terminal (arrow) in an indentation of an odontoblast cell body (OB). b. A closer view of the same nerve terminal, showing immunoreaction products being condensed in cores of the cored vesicles (arrows) in addition to the axoplasm. a: $\times 18,000$, b: $\times 42,000$

Fig. 8. A CGRP-immunoreactive axon terminal in close apposition to the odontoblast process (OP). No synaptic structure is observed between the odontoblast and nerve ending. $\times 13,000$
enveloped by a Schwann sheath, at whose cleft the axolemma abutted on the surrounding basal lamina. A few immunoreactive nerve fibers entirely lacked the Schwann sheath; these naked axons apparently represented terminal portions (Fig. 3). Occasionally CGRP-positive single naked axons, presumably derived from those nerve bundles, terminated near blood vessels.

In the coronal pulp, a number of unmyelinated axons showing immunoreactivity for CGRP ran in the pulp proper. The immunoreactive axons were partially enveloped by a thin Schwann sheath as were those within the radicular nerve bundles. Their morphology was identical to that of the nerves in the subodontoblastic nerve plexus (Figs. 4, 5). Immunoreactive axons occurring adjacent to blood vessels were exclusively of the naked type (Fig. 6).

Numerous CGRP-immunoreactive nerve terminals, all of which were completely devoid of Schwann cell investment, were seen in close association with the cell bodies (Fig. 7) and processes of odontoblasts (Fig. 8). In spite of careful observations, we failed to find any synaptic structures between an odontoblast and either a CGRP-positive or -negative nerve terminal. The CGRP-immunoreactive nerve terminals were filled with numerous small clear vesicles and several cored vesicles, while only a few mitochondria were scattered. Eccentric aggregation of the vesicles was not distinctively observed within the axon terminals. The distribution pattern of the immunoreaction products in the nerve terminals in this area was almost identical to that in other areas of the dental pulp.

DISCUSSION

Although many immunohistochemical studies have dealt with the distribution, morphological features and possible roles of the peptidergic nerves in the pulp (for reviews, AKAI and WAKISAKA, 1990; OLGART, 1990), few reports have been available regarding their ultrastructural configurations. This is due to disadvantages in using hard tissues which make it difficult to decalciy them with suitable preservation of both ultrastructure and immunoreactivity. As far as we know, this is the first report to deal with the detailed ultrastructure of peptidergic nerves in the dental pulp. We concentrated on CGRP-immunoreactive nerve fibers as this neuropeptide seems of especial importance in the tooth, due to its ample distribution in the pulpal nerves.

The present observation confirmed, at the electron microscopic level, that a large number of nerve fibers in the dental pulp were immunoreactive for CGRP, with the major population of them being unmyelinated, and the remaining few myelinated. It was difficult to count the relative proportion of the CGRP-positive and negative axons, since immuno-positive and -negative regions alternately occurred in one single axon (see Fig. 3). In spite of the lack of exact quantitative data, the predominance of unmyelinated axons among the CGRP-containing nerves coincides with previous information that CGRP neurons extending in the dental root ganglia predominantly comprise unmyelinated fibers (for review, ISHIDA-YAMAMOTO and TOHYAMA, 1989).

The CGRP-containing nerve fibers distributed around blood vessels in the dental pulp are regarded as a type of nerve distinct from those involved in sensory transduction (SILVERMAN and KRUGER, 1987), although they are exclusively originated from trigeminal sensory neurons (SILVERMAN and KRUGER, 1987; WAKISAKA et al., 1987). In fact, CGRP is known to be a potent vasodilator (BRAIN et al., 1985), and acts as an enhancer of tachykinin-induced protein extravasation (BRAIN and WILLIAMS, 1985; GAMSE and SARIA, 1985). Physiological experiments have shown that antidiromic stimulation of nerve fibers derived from the trigeminal sensory ganglion induces an increase in the content of pulpal substance P (OLGART et al., 1977), which is colocalized in the CGRP-containing nerve fibers (WAKISAKA et al., 1987), and also increases pulpal blood flow (GAZELIUS and OLGART, 1980). Furthermore, stimulation of trigeminal sensory neurons has demonstrated the antidiromic release of CGRP in vitro (MASON et al., 1984). It is thus reasonable that CGRP is in possible collaboration with substance P released from nerves to act as vasodilator. The present ultrastructural study offers morphological support for this view, in that CGRP-immunoreactive axon terminals in the vicinity of the blood vessel were exclusively devoid of Schwann cell investment and basal lamina.

The majority of nerve terminals in the pulpodental border zone are considered sensory in nature based on experimental and autoradiographic evidence (for review, BYERS, 1984). The nerve terminals in this region are believed to characterize typical free nerve endings filled with numerous mitochondria (GUNJI, 1982). On the other hand, it has been repeatedly reported that CGRP-immunoreactive nerve fibers are abundantly distributed throughout this region. The present study revealed that the CGRP-immunoreactive nerve terminals displayed vesicle-rich features with only a few mitochondria. This characteristic of CGRP-immunoreactive nerve terminals suggests diverse functions for the mitochondria-rich nerve endings. Trigeminal sensory nerves possess the
potential for antidromic release of CGRP (MASON, 1984), and CGRP manifests multiple functions such as trophic effects on vascular smooth muscle cells, fibroblasts and lymphocytes, as well as vascular effects (for review, HOLZER, 1988). Thus, the CGRP-positive nerve terminals associated with odontoblast may also be involved in some active—such as trophic—functions in addition to the receptive function.

The CGRP-immunoreactive axon terminals containing vesicles as seen in the synapse were closely associated with cell bodies and processes of the odontoblasts, and were completely free of Schwann sheath investment. No morphological features reminiscent of a chemical synapse was recognizable between the vesicle-rich CGRP-positive nerve terminals and their associated cells. These findings agree with previous detailed analyses using a conventional transmission electron microscopy which reported that the nerve terminals are merely in close contact with the odontoblasts (TEN CATE and SHELTON, 1966; GUNJI, 1982; for review, BYERS, 1984). It is thus reasonable to assume that CGRP is released from the nerve terminals in a paracrine manner under some condition. A similar arrangement has been demonstrated between CGRP-and/or substance P-positive nerve terminals originating from the sensory neuron and their associated epithelial cells in the skin and taste bud (YAMASAKI et al., 1984; LEE et al., 1985; ISHIDA-YAMAMOTO et al., 1989), suggesting the non-synaptic release of these neuropeptides.

It is notable that immunoreaction products for CGRP were diffusely located in the cytosol of the axoplasm while lacking in membrane-bounded structures, in accordance with the diffuse localization of CGRP in the axoplasm. The localization pattern of the CGRP-immunoreactive products in axons appeared identical to that seen in reactive nerves in other tissues using a pre-embedding method, as was undertaken in the present study (YAMAMOTO and KONDO, 1988; KONDO and YAMAMOTO, 1988; ISHIDA-YAMAMOTO et al., 1989). The finding was interpreted as an artifactual diffusion of antigens or immunoreactive products due to the destruction of the vesicle membrane during the process of specimen preparation (ISHIDA-YAMAMOTO and TOHYAMA, 1989). On the other hand, KONDO and YAMAMOTO (1988) noted that the possibility for diffuse localization of CGRP in the axoplasm in vivo could not be excluded. The hypothesis by the latter is possible, based on current findings as follows: 1) in addition to the diffuse immunoreactive products, cored vesicles containing the immunoreactive products also existed in the same preparations; 2) both immuno-reactive and non-reactive zones could clearly be defined within the individual axons, suggesting little probability for the artifactual diffusion of immunoreaction products within the axoplasm; and 3) CGRP-immunoreactive products were also localized within the preterminal axons where vesicular elements were seldom encountered with conventional electron microscopy.

In conclusion, CGRP was demonstrated to be localized predominantly within the unmyelinated nerve fibers of the dental pulp of rat molars, being located diffusively in the cytosol as well as in the core of large cored vesicles. The CGRP-immunoreactive terminals seemed to function as secretory or effective terminals in addition to receptive terminals, based on current findings that the nerve terminals in close proximity to the wall of blood vessels and the odontoblasts contain numerous, partly immunoreactive, vesicles. Further study is, however, needed in order to elucidate the release mechanism of CGRP from the nerve terminals.

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