Immunocytochemical localization of the neurokinin 1 receptor in rat dental pulp

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Summary. The dentin-pulp complex is a peripheral end-organ supplied by dense sensory nerve fibers. Substance P, a representative neuropeptide widely distributed in the dental pulp, has been reported to play roles in pain transmission and the amplification of inflammation. We analyzed here the expression of the neurokinin 1 (NK1) receptor, preferentially activated by substance P, using immunocytochemistry in rat dental pulp at both the light and electron microscopic levels. Conspicuous NK1 receptor immunoreactivity was found in the odontoblasts; immunolabelings were present at their plasma membrane and endosomal structures, especially in their cytoplasmic processes. Immunoreactions for NK1 receptor were also detectable in a part of the nerve terminals associated with the cytoplasmic processes of the odontoblasts. Furthermore, the endothelial cells of capillaries and post-capillary venules and the fibroblasts were labeled with the NK1 receptor in the subodontoblast layer. These findings suggest that pulpal cells and nerve fibers are targets for substance P that mediate multiple functions, including a vasoactive function and the regulation of vascular permeability as well as the modulation of pain transmission.

Introduction

The dental pulp receives a high density of nerve supply (Byers, 1984) which is believed to be involved in relaying nociceptive information, dentinal fluid dynamics, pulp blood flow regulation, protective reflexes to preserve dental tissues, and wound healing (see review, Byers and Närhi, 1999). These pulp nerve fibers have been shown to be originated from trigeminal ganglion (Byers et al., 1987; Ibuki et al., 1996) and contain certain kinds of neuropeptides, including substance P and calcitonin gene related-peptide (CGRP) (Silverman and Kruger, 1987).

Substance P is widely distributed in the peripheral and central nervous systems and plays an important role in the transmission of nociceptive messages (Nicol et al., 1980). The neurokinin 1 (NK1) receptor, a G-protein coupled receptor with highest affinity for the substance P (Regoli et al., 1988; Ziche et al., 1990) has multiple functions (Otsuka and Yoshida, 1993), including a vasoactive function (Heyeraas and Berggren, 1999; Berggren and Heyeraas, 2000) and the regulation of vascular permeability (Fazekas et al., 1990) as well as the modulation of pain transmission. The effect of substance P depends primarily on the release and availability of the peptide and on the presence of the functional NK1 receptor, which can interact with physiological concentrations. Although the functional receptor has been supposed to be localized to the surface membrane of the target cell, there has been no report demonstrating the ultrastructural localization of the NK1 receptor in the dental pulp. To elucidate the functional site of substance P in the dental pulp, the present study examined the cellular localization of the NK1 receptor in the coronal pulp of the rat molars by immunocytochemistry at the light and electron microscopic levels.
Materials and Methods

Ten adult male Sprague-Dawley rats, weighing 100–160 g, were used in this study. They were treated according to the animal care guidelines of Kyushu University. The animal protocol was reviewed and approved by the Animal Care and Use Committee.

Immunocytochemistry

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and perfused transcardially with a mixture of 4% paraformaldehyde, 0.25% glutaraldehyde, and 0.2% picric acid. The maxillae were dissected out and immersed in the same fixative for 3 h at 4°C. After decalcification in a 5% ethylenediaminetetraacetic acid (EDTA) solution, the tissue blocks were frozen in liquid nitrogen and sectioned in 60 μm thicknesses on a cryostat.

Free-floating sections were then processed for the avidin-biotin peroxidase complex method. Antiserum was raised to a peptide (KTMTESSSFYSNMLA, Takara, Japan) corresponding to the carboxy-terminal 15 amino-acid residues of the rat NK-1 receptor (SPR-393-407) as described previously (Vigna et al., 1994). After treatment with 5% normal goat serum, the sections were incubated with anti-NK1 receptor serum (1:2000) (generous gift of Dr. Vigna), for 48 h at 4°C, followed by two consecutive incubations with biotinylated anti-rabbit IgG (1:200, Vector Laboratory, Burlingame, USA) and peroxidase-conjugated avidin (1:100, Vector Laboratory), each for 12 h at 4°C. The peroxidase reaction was developed for 10–20 min in a 0.05M Tris buffer, pH 7.6, containing 0.02% dianinobenzidine and 0.006% H2O2. The immunostained sections were post-fixed, dehydrated, and embedded in Spurr-resin (Polyscience, Warwington, USA). One μm thick sections were stained with toluidine blue. Ultrathin sections were examined with a JEOL 1210 electron microscope.

Immunocounter experiments

Specificity tests included: 1) omission of the primary or secondary antiserum, or the avidin-biotin peroxidase complex; 2) replacing the primary antiserum with nonimmune rabbit serum; and 3) replacing the primary antiserum to the supernatant of preabsorbed antiserum which was incubated with a concentration of 10^{-5}–10^{-7}M of appropriate synthetic peptide (KTMTESSSFYSNMLA) overnight. No immunoreaction was visible in these control experiments.

Results

NK1 receptor immunoreactivity was detected as brown reaction products in semi-thin sections. Immunoreaction products were found in odontoblasts, endothelial cells of blood vessels, and fibroblasts of the rat dental pulp (Fig. 1a).

The immunoreaction in the odontoblasts was more conspicuous than other cells due to variable labeling intensity, appearing as heterogeneous immunoreaction patterns (Fig. 1b). Light-microscopically, immunoreactions for the NK1 receptor were more intense in the cytoplasm of the cellular processes of the odontoblast than in the cell bodies of the odontoblasts (Fig. 1b). However, the infranuclear region of the odontoblasts was devoid of immunoreaction. We ultrastructurally observed the odontoblast layer and predentin because of the intense immunostaining of the odontoblast and nerve axon. Under the electron microscope, immunoreaction products for the NK1 receptor were observed as electron dense deposits (Fig. 2). NK1 receptor-immunoreaction products were identified in the vesicular and granular structures of the odontoblasts (Fig. 2a). The vesicular structures in the odontoblast processes sometimes appeared to make contact with their plasma membrane (arrowhead in Fig. 2a). The amount of immunoreaction product in odontoblasts was higher in cytoplasmic process (Fig. 2a, c) than in the cell body (Fig. 2a, b). Surface labeling was also observed in the plasma membrane of the odontoblasts, and occasionally the immunopositive invagination of membrane structure was observed (Fig. 2c, d). The cell bodies and processes of the odontoblasts were frequently accompanied with nerve fibers with or without NK1 receptor-immunoreaction (Fig. 2e). These nerve terminals were filled with mitochondria, vesicles and microtubules. Immunoreactivity was found in the axoplasm and axolemma of the nerve terminals which made close contact with the odontoblasts (Fig. 2b, c).

The coronal portion of the pulp received an extensive vascular network beneath the odontoblast layer. The endothelial cells of the post-capillary venules or capillaries were labeled with the NK1 receptor (Fig. 3). Some of the endothelial cells of arterioles were also immunoreacted with the NK1 receptor. Reaction products in the endothelial cells or fibroblasts were found not only close to the plasma membrane but also in the perinuclear region; they were associated with vesicular or vacular structures of various sizes. Surface labeling with the NK1 receptor was found on both the luminal and basal sides of the endothelial cells and was also detected in the invaginations of the plasma membrane (Fig. 3b, c). Sometimes post-capillary venules were accompanied by an axon with the NK1 receptor.
Fig. 1. Photomicrographs showing immunolocalization of the NK1 receptor in the rat coronal pulp. Semithin section counter-stained with toluidine blue. Pre-embedding method. a: NK1 receptor-immunoreaction in the pulp-dentin border zone. Immunoreactions are found in the odontoblasts, endothelial cells, and fibroblasts. b: Higher magnification of the boxed area in a. Odontoblasts (O) are heterogeneously labeled with the NK1 receptor. Note the intense labeling in the cytoplasmic process of odontoblasts in contrast with the weak or scanty labeling in the cell bodies of odontoblasts. D: dentin, PD: predentin. Scale bars: 100 μm (a), 20 μm (b).

Discussion

The present immunostaining was able to demonstrate the subcellular localization of the NK1 receptor in the dentin-pulp complex in normal, non-stimulated conditions at the electron microscopic level, essentially consistent with a previous report by Fristad et al. (1999, 2003), at the light and confocal microscopic levels. The conspicuous labeling was observed in the odontoblasts, most distinctive cells of the dental pulp. The NK1 receptor labeling was localized to the cell surface and vesicle structure, which indicates that NK1 receptor is ready to respond to substance P as a functional receptor. This ultrastructural feature of the localization of the NK1 receptor was also comparable with findings in the trachea (Bowden, 1996), gingiva (Kido et al., 1999), or tempromandibular joint (Kido et al., 2004). This characteristic immunolocalization pattern of the NK1 receptor in the odontoblast and vasculature in the dental pulp indicates that these structures would be targets for substance P, suggesting that this neuropeptide mediates physiological functions in the dentin-pulp complex.

There is no doubt of the involvement of substance P in the nociceptive mechanism in the dental pulp. Substance P-immunoreactive nerve fibers are widely distributed in the dental pulp, and some of them enter the predentin beyond the odontoblast layer to terminate as free nerve endings there (Wakisaka et al., 1985). In addition, substance P expression in pulpal nerves in human teeth suffering pain has been reported to show higher amounts than in healthy teeth (Rodd et al., 2000). The present immunocytochemical observation showed that the conspicuous labeling was found in the cytoplasmic process of the odontoblast rather than the cell body. This finding indicates that substance P may take part in sensory mechanism in the predentin area via the NK1 receptor. Probably, stimulation to the tooth causes a release of substance P from pulp nerve fibers to induce NK1 receptor internalization in both nerves and odontoblasts.

Previous in vitro studies have shown the possibility that substance P controls the cellular activity; substance P has a stimulating effect on fibroblasts (Nilsson et al., 1985; Ziche et al., 1990; Kahler, 1993) and induces the synthesis and expression of interleukin-1 or interleukin-6 and tumor necrosis factor-β in human dental fibroblasts (Yamaguchi et al., 2004). Furthermore, Jacobson and Heyeraas (1996) demonstrated that inferior alveolar nerve axotomy or capsaicin-induced denervation significantly reduced dentin formation at the pulp horn and at the central pulp floor compared with controls. Combined with these findings, the topographic relationship between nerve fibers and odontoblasts as well as the expression of the NK1 receptor in fibroblasts as demonstrated in this study suggest that the
Fig. 2. Legend on the opposite page.
Fig. 2. a–e: Immunoelectron micrographs showing the NK1 receptor in odontoblast and axon profiles. The cytoplasmic process of the odontoblast labeled with the NK1 receptor. Immunoreactivity is found in the vesicular (arrow) and vacuolar structures of the cells. NK1 receptor immunoreactivity is found in the axon profiles between odontoblasts (b). The axon profile is filled with mitochondria, vesicles, and microtubules. Note the intense labeling in the axolemma of the nerve (b, e). NK1 receptor immunoreactive axon profiles make close contact with the odontoblast process. d: The higher magnification indicated by square in e. Note the surface labeling invagination in the odontoblast process (double arrows). Od: odontoblast, OP: odontoblast process. Bars = 500 nm (a, b), 1 μm (c, d), 400 nm (e)

Fig. 3. a–c: Immunoreaction for the NK1 receptor in the endothelial cells in the subodontoblastic layer. NK1 receptor labeled endothelial cells (E) beneath odontoblast layer. Arrow indicates the NK1 receptor-immunoreactive axon profile (a). Surface labeling with the NK1 receptor is found on both basal (b) and luminal (c) side of the endothelial cells (arrowheads). Pulpal fibroblast (F) contains immunoreaction products in its cytoplasm. L: lumen. Scale bars: 1 μm
involvement of substance P in the progress of inflammation and dentin formation is regulated by sensory input.

Substance P is famous for its vasoregulatory functions including increases in blood flow or plasma extravasation (Lembeck and Holzer, 1979; Otsuka and Yoshida, 1993; Kondo et al., 1995). Nerves with substance P are distributed to the dental pulp (Olgaard et al., 1977; Wakisaka et al., 1985). Kim et al. (2005) demonstrated that the effect of NK1 receptor antagonists attenuated substance P-induced vasodilatation of the pulp. Furthermore, sensory nerves in the tooth are responsible for the increase in pulp interstitial fluid pressure and blood flow (Jacobsen and Heyeraas, 1996; Berggren and Heyeraas, 2000). The NK1 labellings in the endothelial cells at the subodontoblast layer were associated with the plasma membrane and vesicular structures as endosomes, indicating that the NK1 receptor is ready to bind to the ligand and then be internalized into the endosomes. The NK1 receptor localized both on the cell surface membrane and endosomal structure may represent the endocytosis of substance P and the NK1 receptor to regulate the vasculature and interstitial dentin-pulp flow.

References


