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ABSTRACT

Hypersensitivity to thermal and mechanical stimuli can occur in painful pulpitis. To explore the neuro-anatomical basis of heat and mechanical sensitivity, we evaluated expression of TRPV1 (heat) and TRPV2 (heat/mechanical) channels in the cell bodies and terminal arborizations of neurons that innervate the dental pulp (DP) and periodontal tissues (PDL). We report that ~50% of trigeminal ganglion (TG) neurons retrogradely labeled from the DP express TRPV2, and this was significantly greater than the general expression of this channel in the TG (15%) and slightly more than what is expressed in the PDL by retrograde labeling (40%). The TRPV1 receptor, however, was less prevalent in neurons innervating the DP than their general expression in the TG (17% vs. 26%) and was more extensively expressed in neurons innervating the PDL (26%). Co-labeling studies showed that 70% of neurons that innervate the DP are myelinated. Approximately 1/3 of the retrogradely labeled neurons from the DP were calcitonin-gene-related-peptide-positive (peptide-expressing), but very few expressed the IB4 marker of non-peptidergic unmyelinated afferents. These findings suggest that the DP has a unique neurochemical innervation with regard to TRP receptor expression, which has significant implications for the mechanisms contributing to odontogenic pain and management strategies.

KEY WORDS: pain, TRPV2, TRPV1, dental pulp, heat.

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Differential TRPV1 and TRPV2 Channel Expression in Dental Pulp

INTRODUCTION

Applying a heat stimulus to the tooth evokes pain by activating nociceptors that innervate dental pulp (DP), and these nociceptors in turn engage second-order neurons in the trigeminal nucleus (Chattipakorn *et al.*, 2001). Inflammation of the dental pulp is associated with thermal and mechanical hypersensitivity, *i.e.*, exaggerated sensitivity to cold or heat and enhanced mechanical activation of neurons in the dentinal tubules *via* hydrodynamic fluid movement (Närhi *et al.*, 1982; Pau *et al.*, 2005; Owatz *et al.*, 2007). For example, heat hyperalgesia occurs in advanced painful cases of irreversible pulpitis and is associated with inflammatory and hyperemic histological changes in the pulp (Dachi, 1965; Cecic *et al.*, 1983). The biological mechanisms of intra-oral thermal and mechanical allodynia and hyperalgesia, however, remain unclear. The hydrodynamic theory suggests that there is an important mechanism for indirect temperature-evoked activation of pulpal neurons, but it is likely that direct thermal activation of these neurons is also relevant (Brannström, 1986).

The discovery of transient receptor potential (TRP) ion channels has contributed greatly to our understanding of molecular mechanisms that mediate distinct sensory modalities (Patapoutian *et al.*, 2009; Stucky *et al.*, 2009). TRPV1 and TRPV2 are two TRP channels originally described as heat-sensitive (Caterina *et al.*, 1997, 1999). Heat (> 43°C), pH, capsaicin, and inflammatory mediators activate or sensitize TRPV1, and the expression of TRPV1 is influenced by inflammatory mediators and nerve injury (Tominaga *et al.*, 1998; Cesare *et al.*, 1999; Caterina *et al.*, 2000; Ji *et al.*, 2002). Deletion of the TRPV1 receptor through both genetic and chemical means has confirmed a behavioral contribution of TRPV1 to heat sensitivity (Caterina *et al.*, 2000; Cavanaugh *et al.*, 2009). TRPV2, by contrast, has a much higher thermal activation threshold (> 52°C) (Caterina *et al.*, 1999; Lewinter *et al.*, 2004). Although TRPV2 was originally considered to process high-intensity heat, whether its *in vitro* properties are representative of its *in vivo* function has not yet been established. In fact, deletion of TRPV2 does not alter heat pain processing (Park *et al.*, 2008). Other studies suggest that TRPV2 responds to membrane stretch (in aortic myocytes) and may serve a more general mechanosensitive function (Muraki *et al.*, 2003; Lawson *et al.*, 2008; Shibasaki *et al.*, 2010).

To date, studies have demonstrated TRPV1 and TRPV2 receptors in neurons innervating dental pulp, but neither the relative expression of these channels nor their association with molecular markers of subpopulations of trigeminal ganglion neurons has been analyzed in detail (Chaudhary *et al.*, 2001; Ichikawa and Sugimoto, 2001; Stenholm *et al.*, 2002; Morgan *et al.*, 2005). Here we use retrograde labeling methods combined with immunohistochemistry to compare the expression of TRPV1 and TRPV2 in two intra-oral heat and mechanosensitive tissues, namely, the dental pulp and periodontal tissues.

This analysis established the relative contributions of neurochemically distinct TG neurons to pulpal and periodontal TRP channel innervation.

MATERIALS & METHODS

Experimental Animals

All studies were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the National Institutes of Health Guide and Public Health Service Policy on humane care and use of laboratory animals and the recommendations of the International Association for the Study of Pain. Two rats were housed per cage and maintained on a 12-hour light/dark schedule with *ad libitum* access to food and water. Twelve rats were used to complete these studies and none were dropped from the study.

Retrograde Labeling of Trigeminal Ganglion Neurons

The procedures used for retrograde (back) labeling of pulpal neurons, without inducing significant inflammation and necrosis, involve local application of the tracer Fluoro-Gold to dentin (Pan *et al.*, 2003). Although it is unlikely that this tracer labels all trigeminal ganglion neurons that innervate the pulp, Fluoro-Gold likely reveals a more representative population than do other tracers (Zelevansky *et al.*, 2010). *Dental Pulp (DP)*: Eight male adult Sprague-Dawley rats (200-250 g) were deeply anesthetized with a combination of ketamine HCl (60 mg/kg) and xylazine (7.5 mg/kg) by the intra-peritoneal route. After depth of anesthesia was confirmed, the jaw of each animal was propped open by means of a standard set of cotton forceps placed between the maxillary and mandibular molars. Using a ¼ round bur, we next made a shallow cavity into the dentin of the lingual surfaces of the left first and second maxillary molars and the mesial surface of the left maxillary first molar. Magnification and illumination *via* high-powered loupes and headlight allowed for adequate visualization. The cavity preparations were washed with sterile saline and dried with cotton pellets and air. The smear layer was removed from the cavities by etching with a gel containing 32% phosphoric acid (Young Dental, Earth City, MO, USA). The cavity was washed and dried again, and then a single 5% drop of the retrograde tracer Fluoro-Gold (Fluorochrome, Denver, CO, USA) was placed onto each cavity by means of a 10 µL Hamilton Syringe, and allowed to dry for 1-2 min. *Periodontal Tissues (PDL)*: In a separate set of experiments with 4 adult male rats, we labeled periodontal tissues by injecting ~10 µL of 5% Fluoro-Gold into the periodontal ligament space located between the gingiva and the 1st and 2nd left maxillary molars, using an insulin syringe at multiple sites around the teeth.

Tissue Preparation and Immunocytochemistry

Animals were sacrificed by overdose with sodium pentobarbital (100 mg/kg) and perfused intracardially with 50 mL of 0.1 M phosphate-buffered saline (PBS), followed by 500 mL of 10% formalin in 0.1 M sodium phosphate buffer. The trigeminal ganglia and maxillary jaws were collected, post-fixed for 4 hrs, and

then cryoprotected overnight in 30% sucrose in 0.1 M PB. Maxillae were decalcified in 10% EDTA (pH 7.6) for 2 to 3 wks. Tissues were sectioned in a cryostat (maxillae at 40 µm and TG at 18 µm) and thaw-mounted onto Superfrost Plus Microscope Slides (Sigma-Aldrich, St. Louis, MO, USA).

Tissue sections were incubated for 60 min at room temperature in a blocking solution of 5% normal goat serum in PBS with 0.3% Triton X-100. Slides were incubated overnight at 4°C with a rabbit anti-TRPV1 (1:5000) or rabbit anti-TRPV2 (1:1000) antibody, both kindly provided by Dr. David Julius (UCSF). Both antibodies were previously characterized for specificity (Caterina *et al.*, 1999; Lewin *et al.*, 2004). Importantly, the TRPV1 antibody does not immunostain neurons in TRPV1-null mice. Sections were then incubated with either Alexa Fluorophore 488 or 546 goat anti-rabbit (Molecular Probes, Eugene, OR, USA; 1:700) for 2 hrs at room temperature. For double-label studies, antibodies used included mouse anti-CGRP (1:500; Sigma-Aldrich) and mouse anti-neurofilament 200 (1:10,000; Sigma-Aldrich). The lectin IB4 was visualized with biotinylated IB4 (1:500; Vector Laboratories, Burlingame, CA, USA) and Alexa Fluorophore 594 conjugated to streptavidin (1:1000; Invitrogen, Carlsbad, CA, USA). Sections were observed with either a fluorescence (Nikon Eclipse) or confocal microscope (Zeiss LSM510). Image J was used to count cells and measure cell diameters.

Data Analysis

The results of cell counting were recorded in an Excel spreadsheet and then transferred to STATA (version 10) for analysis. Figures were generated using GraphPad Prism (ver. 5.0b). Standard descriptive statistics were used. For comparison between groups, we used an unpaired two-way *t* test. Significance was at the $p < 0.05$ level.

RESULTS

Expression of TRPV1 and TRPV2 in Fluoro-Gold-labeled Pulpal and Periodontal Neurons of the Trigeminal Ganglia

We first evaluated the percentage of rat TG neurons that express the TRPV2 and TRPV1 receptors. Consistent with most other studies, we found that TRPV2 is expressed in about 15% of all neuronal cell bodies of the TG and TRPV1 in about 25% (Table). We then determined their tissue-specific distribution by retrograde labeling neurons that innervate the dental pulp (DP) and periodontal tissues (PDL) using Fluoro-Gold (FG). To ensure the feasibility of making injections that selectively target the DP and PDL, we examined the spread of FG in decalcified maxilla sections. Appendix Fig. 1 illustrates that it is indeed possible to localize injections, making our subsequent analysis of the innervation of the DP and PDL possible.

To determine the tissue-specific neuronal distribution of TRPV1 and TRPV2 receptors, we counted the co-expression of TRPV1 and TRPV2 immunoreactivity in FG-labeled neurons that innervate the DP and PDL. We found that 50% (Table) of all DP cell bodies expressed TRPV2 (Figs. 1a, 1e, 1i, 1c, 1g, 1k),

Figure 1. TRPV2 was highly expressed in the cell bodies of neurons innervating the dental pulp. Sections of trigeminal ganglia containing pulpal neurons that were retrogradely labeled with Fluoro-Gold were evaluated for TRPV2 expression. Tailed arrows point to cell bodies that expressed Fluoro-Gold and immunoreactivity for the indicated antigen. Arrowheads highlight cells that expressed Fluoro-Gold and were immunonegative for the indicated antigen. Fluoro-Gold-positive cells are shown in **Panels c, g, and k**. TRPV2 was expressed in pulpal neurons (**Panels a, e, i**) that almost always co-expressed NF200 (**Panel b**) and sometimes CGRP (**Panel f**). Overlay images are also included (**Panels d, h, l**). The non-peptidergic nociceptive marker IB4 was not found in pulpal neurons (**Panel j**). All scale bars = 200 μm .

Table. Comparison of TRPV1 and TRPV2 Expression in Neurons of the Dental Pulp (DP) with Expression of These Channels in the Periodontal Tissues (PDL) and Trigeminal Ganglion (TG)

	DP	PDL	TG	p-value
TRPV2	50.2 $\pm$ 11.9 n = 8 (817)	39.9 $\pm$ 10.3 n = 4 (563)	14.5 $\pm$ 1.8 n = 4 (2082)	DP vs. PDL: p = 0.17 DP vs. TG: p = 0.0002 PDL vs. TG: p = 0.003
TRPV1	16.9 $\pm$ 6.8 n = 8 (682)	25.8 $\pm$ 3.6 n = 4 (626)	25.8 $\pm$ 2.6 n = 4 (2267)	DP vs. PDL: p = 0.04 DP vs. TG: p = 0.03 PDL vs. TG: p = 0.99

Unpaired two-tailed *t* tests were used to determine the difference between groups. The average and SD represent the frequency of expression averaged by rat, n = number of rats used in analysis, and (x) = the number of Fluoro-Gold-positive profiles counted *per* analysis. A statistically significant difference between groups is considered at the p < 0.05 level.

while only 17% expressed TRPV1 (Figs. 2a, 2e, 2i, 2c, 2g, 2k). There were slight differences in the innervation of the PDL relative to DP, with 40% of all retrogradely labeled cell bodies expressing TRPV2 and 26% expressing TRPV1 (Table). These

results indicate that the DP and PDL innervation is dominated by afferents that express TRPV2, at least when compared with TRPV1. The relatively sparse TRPV1 innervation of the DP indicates that the DP is likely less responsive to heat *via*

Figure 2. TRPV1 is infrequently expressed in the cell bodies of neurons innervating the dental pulp. Sections of trigeminal ganglia containing pulpal neurons that were retrogradely labeled with Fluoro-Gold were evaluated for TRPV1 and TRPV2 expression. Tailed arrows point to cell bodies that expressed Fluoro-Gold and/or immunoreactivity for the indicated antigen. Arrowheads highlight cells that expressed Fluoro-Gold and were immunonegative for the indicated antigen. TRPV1 was infrequently expressed in pulpal neurons (**Panels a, e, i**), but even these few were often NF200-positive (**Panel b**) and sometimes CGRP-positive (**Panel f**). The non-peptidergic nociceptive marker IB4 was not found in pulpal neurons (**Panel j**). All scale bars = 200 μ m.

TRPV1-mediated mechanisms than other tissues of the trigeminal system, including periodontal tissues.

Cell diameter measurements showed that the TRPV2 innervation of the DP derives from neurons that ranged between 71.2 and 2577.8 μ m² (median = 868.6). The vast majority of these (87%) are of medium and large diameter (> 400 μ m²) (Fig. 3a). By contrast, the TRPV1 innervation is derived from somewhat smaller-diameter neurons, 66.2–2256.2 μ m² (mean = 741.4). Again, as for the TRPV2 neurons, the great majority of these (82%) are medium and large diameter neurons (Fig. 3b).

Co-localization of TRPV1 and TRPV2 with Neurochemical Markers of Nociceptors that Innervate the DP

We found that approximately 1/3 of FG-labeled pulpal neurons are peptidergic, *i.e.*, co-label for CGRP (37 $\pm$ 11% of 486 FG

cells counted; Figs. 1f–1h, Figs. 2f–2h). Of particular interest, many of these pulpal cell bodies give rise to myelinated axons, since they also express NF200, a neurofilament marker of myelinated axons (71 $\pm$ 13%, of 471 FG cells counted; Figs. 1b–1d, Figs. 2b–2d). Interestingly, we found that few FG-labeled cells bound the lectin IB4, which is a marker of non-peptidergic subpopulation of unmyelinated C-fibers (1 $\pm$ 1.7%, of 543 FG cells counted; Figs. 1j–1l, Figs. 2j–2l). In co-labeling studies, we found that about 1/3 of the FG-labeled TRPV2-ir cells from DP expressed CGRP (33%, 49/150; Figs. 1e–1h). This is considerably less than the pulpal TRPV1-ir cells, of which greater than half co-labeled with CGRP (60%, 23/38; Figs. 2e–2h). Finally, we found that the great majority of FG-labeled cells that express TRPV1 and TRPV2-ir are myelinated, as demonstrated by their high degree of co-localization with NF200 (TRPV1, 68%, 17/25; Figs. 2a–2d; TRPV2, 83%, 104/126; Figs. 1a–1d).

Direct Visualization of TRPV1 and TRPV2 Axonal Arborizations in the Dental Pulp

We also examined the arborization of TRPV1 and TRPV2 axons in the dental pulp of rodent maxillary molars. We found that TRPV2 receptor immunoreactivity was abundantly expressed in NF200-positive axonal profiles in the dental pulp (Appendix Figs. 2a-2c). This supports the conclusions drawn from the Fluoro-Gold retrograde-labeling studies. We were not able to demonstrate convincingly any TRPV1 receptor expression in the dental pulp using immunohistochemistry (data not shown). This was expected, given the low expression of TRPV1 in cell bodies that innervate the DP (Table).

DISCUSSION

In this study, we found that the TRPV2 receptor is frequently expressed in pulpal sensory neurons relative to their expression in trigeminal ganglia as a whole. By contrast, TRPV1 is underrepresented in pulpal sensory neurons relative to their expression in the trigeminal ganglia. These observations support the contention that the dental pulp is a uniquely innervated nociceptive tissue and may, in fact, have a limited capacity to detect heat *via* TRPV1, at least under uninjured conditions. This finding could be related to the clinical observation that heat is an unreliable stimulus to test pulp vitality (Petersson *et al.*, 1999). Although the IB4-positive population of neurons is heat-sensitive (Albers *et al.*, 2006), these afferents are quite limited in the dental pulp, and likely contribute minimally to heat sensitivity *in vivo* (Cavanaugh *et al.*, 2009). Taken together with the fact that TRPV2 is no longer considered a major transducer of noxious heat stimuli, our results suggest either that very few TRPV1 afferents are required to mediate heat sensitivity of the DP, or that as-yet-unidentified heat transducers or mechanisms are involved.

Although the level of TRPV1 in the pulp was low relative to periodontal tissues and trigeminal ganglia, there is no question that the TRPV1 innervation is functional. Thus, capsaicin, which specifically targets TRPV1, can stimulate CGRP release from isolated dental pulp (Gibbs and Hargreaves, 2008; Fehrenbacher *et al.*, 2009), and transdermal application of capsaicin to incisor dental pulp can evoke nocifensive behavior in rats (Chidiac *et al.*, 2002). Finally, electrophysiological studies have identified capsaicin-sensitive fibers in dental pulp (Ikeda *et al.*, 1997; Chaudhary *et al.*, 2001). However, other studies provide support for the hypothesis that TRPV1 is not a major contributor to pulpal nociception. Thus, depletion of capsaicin-sensitive fibers by neonatal administration of capsaicin has little effect on noxious electrical and mechanical tooth pulp stimulation, indicating a relatively minor role for capsaicin-sensitive fibers (Tal, 1984; He *et al.*, 2000), at least for these modalities of pulpal nociception. Conceivably, TRPV1-expressing pulpal neurons are not major contributors to nociceptive processing in non-injured dental pulp, but take on a more critical role after injury, namely, in the setting of pulpal inflammation and neural degeneration.

Given the high expression of TRPV2 in the dental pulp and the fact that this receptor is unlikely to contribute to heat detection, it

Figure 3. Cell size distribution analysis of TRPV1 and TRPV2 expressing pulpal neurons in the trigeminal ganglia.

is of great interest to determine its function in the DP. One intriguing possibility is that TRPV2 receptors are expressed on mechanosensitive nociceptors, as recently demonstrated in an electrophysiological study of somatic afferents (Lawson *et al.*, 2008). Since the dental pulp is densely innervated with mechanosensitive nociceptive fibers, and TRPV2 is concentrated in myelinated afferents, it is conceivable that the two populations overlap, and that TRPV2 contributes to the mechanosensitivity of the pulpal afferent (Muraki *et al.*, 2003). Although pharmacological antagonism of TRPV2 is not yet possible, TRPV2 knock-out mice are now available, so that functional studies in the pulp can be performed.

From this analysis, we conclude that afferents that innervate dental pulp and periodontal tissues have a neurochemical signature manifest in the differential expression of the heat-sensitive channel TRPV1 and the purported heat/mechano-sensitive channel TRPV2. The expression pattern of these receptors in peripheral afferents of these tissues likely contributes to the high sensitivity of these tissues to detection of innocuous as well as noxious thermal and mechanical stimuli. Clearly, future studies should address the distribution of other TRP channels (*e.g.*, the cold-sensitive TRPM8 channel) that likely defines a non-overlapping population of pulpal afferents.

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