Capsaicin Receptor VR1 and ATP Purinoceptor $P2X_3$ in Painful and Nonpainful Human Tooth Pulp

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Aims: *To investigate the levels of the capsaicin or vanilloid receptor-1 (VR1) and the ATP-gated purinoceptor $P2X_3$ in painful and nonpainful human tooth pulps.* ***Methods:*** *Immunohistochemistry with specific antibodies and image analysis was used to quantify VR1- and $P2X_3$-positive nerve fibers in painful (n = 13) and nonpainful (n = 33) human tooth pulps, and VR1 immunoreactivity was compared with immunoreactivity for the structural neuronal marker peripherin.* ***Results:*** *Strong VR1-like immunoreactivity was documented for the first time in dental pulp neurons. Weaker $P2X_3$-like immunoreactivity was also detected in fewer nerve fibers. The ratio of VR1 to peripherin immunoreactivity was not significantly different between nonpainful and painful tissues (mean ± SE % area of VR1 to peripherin; nonpainful 53.4 ± 4.7%, n = 33; pulpitis 35.1 ± 7.1%, n = 13; P = .07).* ***Conclusion:*** *The presence of VR1 and $P2X_3$ in fibers of human tooth pulp suggest that they may play a role in perception of dental pain, but further studies, including quantitation of their ligands, are necessary to elucidate any role they may play in pathophysiologic states.* J OROFAC PAIN 2003;17:245–250.

Key words: human tooth pulp, $P2X_3$, vanilloid receptor

Nociceptors express ion channels and receptors that respond to noxious stimuli and so are involved in the initiation of pain. There are several main families, which include sodium, potassium, proton, capsaicin (heat), and adenosine triphosphate (ATP)-sensitive (purinoceptor) groups.[1] The vanilloid receptor-1 (VR1) and purinoceptor $P2X_3$ are 2 key molecules in pain mechanisms.[2,3] A number of factors produced by inflammation, ischemia, or injury can activate these 2 receptors. VR1 is sensitive to capsaicin, noxious heat (> 46°C), eicosanoids, leucotriene B, and protons. $P2X_3$ is activated by ATP, which itself can elicit pain when directly applied to blister bases in humans,[4] and can increase nociceptive behavior in animals. Studies on null mutant mice have elucidated the sensory roles of VR1[5] and $P2X_3$.[6,7] The potential role of VR1 and $P2X_3$ in mediating nociception is further supported by their increased expression in inflamed human bowel.[8,9]

Previous dental studies have reported $P2X_3$ receptor immunoreactivity in rat dental pulp[10] and taste buds,[11] and 1 study has reported the presence of $P2X_3$ in healthy human dental pulp.[12]

Capsaicin-sensitive fibers have been reported in the feline dental pulp,[13] and a recent report identified VR1 fibers in rat dental pulp.[14] Our study aimed to investigate the levels of VR1 and $P2X_3$ in painful and nonpainful human dental pulp.

Materials and Methods

Tissues

The study involved 46 patients who were scheduled for third molar extraction at Guy's Dental Institute, London, and who consented to the study in accordance with the local ethics committee. The teeth scheduled for extraction were tested 1 hour prior to surgery with an electric pulp tester (analytic technology constant current at the midbuccal surface of the tooth) to ascertain the neural vitality of the dental pulp. A pain history was recorded and the patients were divided into 2 groups, those with existing pain and those with no history of existing pain. Indications for extraction included pericoronitis (infection of the tissues around the tooth) for the healthy, nonpainful teeth (n = 33 patients, 13 male and 20 female), and painful pulpitis (infection of the dental pulp) due to coronal caries (n = 13 patients, 4 male and 9 female; pain duration 0.5 to 3 months, mean 2.23 months). All the teeth were removed by a standard buccal approach under local or general anesthesia. Subsequent to the extraction process (with a time limit of 5 minutes), the teeth were sectioned vertically with a water-cooled drill and the pulp placed onto a sterile card, to maintain orientation of the specimen. All specimens were snap-frozen and stored at –70°C prior to study.

Antibodies and Peptides

An affinity purified rabbit antibody that was raised against a synthetic amino acid peptide sequence of human VR1 (Roche Bioscience) and an antibody against a C-terminal fragment of $P2X_3$ (Roche Bioscience) were used as described previously.[9] Two different neuronal markers were also used. One of these was a monoclonal antibody that reacts to the phosphorylated polypeptide subunits 200 and 70 kDa, which are generally present in neurofilaments (clone 2F11, Dako; see Discussion) but does not react with other intermediate filament proteins. This antibody labels neurons, neuronal processes, and peripheral nerves, as well as sympathetic ganglion cells. Another monoclonal antibody specific for peripherin (Clone PJM50, Novo), a Class III intermediate filament subunit of 57 kDa, also found in both the peripheral and central nervous systems (although it is concentrated as its name suggests, in the neurons of peripheral ganglia and their processes) was also used. Human dorsal root ganglion (DRG) and urinary bladder were collected, as previously described, and used as a positive control for VR1 and $P2X_3$ staining.[15]

Immunohistochemistry

Frozen sections (10 µm) of tooth pulp were thaw-mounted onto poly-L-lysine (Sigma Chemicals)-coated slides and post-fixed for 30 minutes with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Sections were then dehydrated in industrial methylated spirit (IMS) containing 0.3% hydrogen peroxide for a further 30 minutes, washed, and incubated with appropriate primary antibody overnight. Sections were washed and incubated with biotinylated goat anti-rabbit (or mouse) and ABC complex (Vector Laboratories). Sites of antibody attachment were revealed through the use of the nickel ammonium sulphate-intensified diaminobenzidine reaction method. Positive reaction product was grey/black and preparations were counterstained to give red nuclei (Neutral Red 0.1% w/v). Negative controls included replacement of primary antibodies with normal rabbit serum or omission of primary antibody. Antibody specificity was tested by pre-incubating primary antibody with decreasing dilutions of the relevant peptide for 2 hours. Peptide concentrations initially at 0.2 mg/mL were serially diluted (1:10 each time) to give a total of 5 different peptide concentrations.

Image Analysis

VR1 and peripherin immunoreactivity in fibers was quantified by computerized image analysis (Seescan Cambridge). Images were captured via video link to an Olympus BX50 microscope (×40 objective) and scanned by computer. Setting grey-level detection limits at threshold highlighted positive immunostaining, and the area of highlighted fibers was obtained as % area of the field scanned. Scanning was performed for a minimum of 5 random fields per tissue section orientated longitudinally. Results are expressed as the percentage of the mean VR1 to peripherin fibers in each field. The Mann-Whitney test was used to compare ratios between groups. The Spearman rank correlation coefficient was used to test the correlation

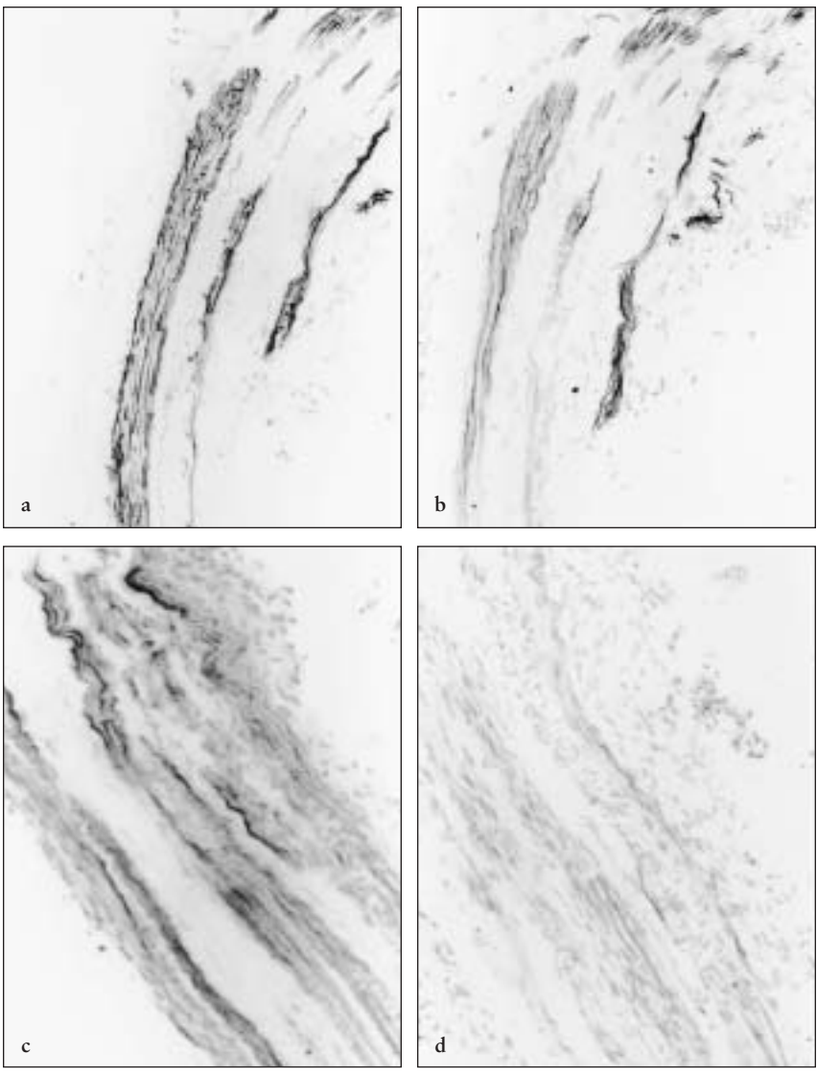

Fig 1 Photomicrographs (×200) of immunoreactivity of human dental pulp to neurofilament *(a)*, peripherin *(b)*, VR1 *(c)*, and $P2X_3$ *(d)*.

between peripherin and VR1 reactive areas. $P < .05$ was considered to reflect statistical significance.

Results

Immunohistochemistry

Strong staining of both neurofilament (Fig 1a) and peripherin (Fig 1b) nerve fibers was seen in all tooth pulp sections examined. In general, the number of neurofilament-positive fibers was evenly distributed; thick bundles far exceeded the thinner peripherin-positive bundles. Peripherin immunoreactivity was similar for nonpainful and painful groups (Figs 1b and 2). There was no differential expression of neurofilament or peripherin between genders or in association with the duration of pain.

VR1 Immunoreactivity

We observed VR1 immunoreactivity in thin varicose nerves in the subodontoblastic plexus in healthy and painful teeth; the staining was very intense and, like peripherin-positive fibers, was evenly distributed (Figs 1c and 3). Image analysis of VR1 immunoreactivity showed significant correlation to peripherin for all tissues ($r = 0.63$, $P = .02$). The ratio of VR1 to peripherin immunoreactivity was not significantly different between nonpainful and painful pulps (% area of VR1 to peripherin; nonpainful 53.4 ± 4.7%, n = 33; painful 35.1 ± 7.1%, n = 13; $P = .07$). There was no apparent difference between the nonpainful and painful teeth with respect to the intensity of VR1 immunoreactivity in pulpal nerves. There was also no apparent association between VR1 expression and the duration of pain or gender. The VR1

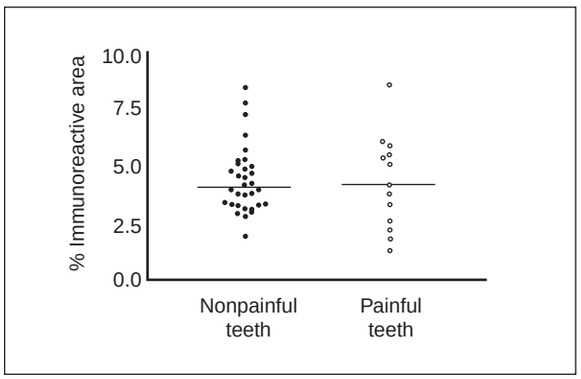

Fig 2 Peripherin immunoreactivity in human dental pulpal nerves from healthy, nonpainful teeth and carious painful teeth. Each circle represents the mean percentage of 5 random fields. The median is indicated by the horizontal lines.

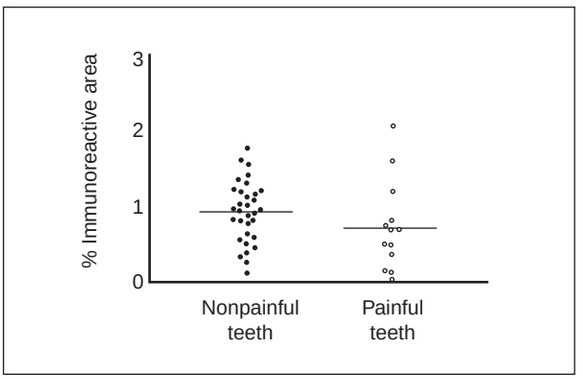

Fig 3 VR1 immunoreactivity in human dental pulpal nerves from healthy, nonpainful teeth and carious painful teeth. Each circle represents the mean percentage of 5 random fields. The median is indicated by the horizontal lines.

staining was completely abolished when the primary antibody was pre-incubated with VR1 peptide at high peptide concentrations, but reappeared as the peptide concentration was decreased.

$P2X_3$ Immunoreactivity

$P2X_3$ immunoreactive fibers were found throughout the pulp, but the expression was relatively weak, so no attempt at image analysis was made (Fig 1d). $P2X_3$ immunoreactivity was stronger in the positive control tissues (human DRG and urinary bladder).

Sampling

Examination of the relationship of the neuron receptors to odontoblasts or to dental carious cavities was not possible, as decalcification was necessary and these relationships can only be evaluated in intact crowns. The mechanical removal of the pulp results in the absence of odontoblasts, as they are stripped away and left in the dentin tubules.[16] The pulp morphology after detachment from the crown was well preserved, and the pulp horns were prominent.

Discussion

This study demonstrates, for the first time, that numerous VR1 immunoreactive nerve fibers exist in the human dental pulp, and are similar in appearance to peripherin-positive fibers. There was no difference in VR1-expressing pulpal fibers, in proportion to peripherin-positive fibers, between painful and nonpainful teeth. $P2X_3$ immunoreactivity was less evident than VR1 immunoreactivity throughout the pulp.

Neurofilament and Peripherin

It is known that the majority of pulpal fibers originates in the trigeminal ganglion. The pulpal nerves are predominantly sensory fibers of C and Aδ type[17] and react with neurofilament and peripherin.[12,18] Peripherin immunostaining was in accord with a previous study, which showed that the subodontoblastic plexus contains predominantly C and A-delta nociceptive fibers.[19]

VR1

The VR1 immunoreactivity was observed in thin varicose fibers, in support of a role in dental pain. These fibers may correspond to A-delta or C fibers, which have been shown to respond to capsaicin in the cat dental pulp.[13] Expression of VR1 confers capsaicin sensitivity and heat sensitivity at noxious temperatures.[20] There is a correlation between capsaicin sensitivity and noxious heat sensitivity in DRG neurons.[21] Once activated, capsaicin-sensitive neurons evoke a sensation of burning pain and the release of sensory neuropeptides CGRP and substance P from peripheral terminals, which subsequently induce neurogenic inflammation; both neuropeptides have been reported to be elevated in inflamed human dental pulp.[22,23] Several mediators associated with inflammation may increase nerve growth factor (NGF), which regulates the expression of capsaicin sensitivity of human sensory neurons.[24]

There appeared to be no difference in the intensity of the immunoreactivity to VR1 in nerve fibers in the pulpitis group compared to controls. Clinically, teeth with a powerful pulpitis are more heat sensitive, but VR1 immunoreactivity appeared not to be increased in this cohort of teeth manifesting painful pulpitis. There was no relationship between the duration of pain and the expression of VR1 in this study. It is at present unclear why VR1 immunoreactivity was unchanged in this study, since other studies report an increase of neuropeptide CGRP and substance P levels in the rat[25] and human,[26,27] given that the expression of VR1 and these neuropeptides are regulated by NGF. A number of possible explanations need to be considered. The lack of any observed change may be due to the inflammatory changes being relatively acute; more chronic and severe changes may be necessary for increased expression of VR1, as we have demonstrated in inflamed human bowel.[8] Alternatively, a detectable increase of VR1 may require both increased NGF and glial derived neurotrophic factor expression, as they are both known to regulate capsaicin receptor expression, whereas substance P and CGRP are regulated by NGF alone. Technical aspects may also be relevant. It may be easier to detect an increase of the sensory neuropeptides than an ion channel such as VR1 by immunocytochemical methods. Other quantitative techniques, such as Western blot, may be necessary to demonstrate any significant changes. Studies of factors that may trigger or sensitize VR1-expressing nerve terminals also deserve consideration.

$P2X_3$

The purinoceptor $P2X_3$ is a cation channel that is activated extracellularly by ATP. Electrophysiologic responses of rat tooth-pulp nociceptors to ATP are characteristic of $P2X_3$.[12] $P2X_3$ are upregulated in the rat trigeminal nerve ganglion after inferior alveolar nerve injury,[28] and there is a report of upregulated $P2X_3$ expression in the DRG after chronic constriction injury to the sciatic nerve.[29] $P2X_3$ immunostaining has been previously reported in human dental nerve fibers.[12] The relatively weak expression of $P2X_3$ in this study suggests a differential distribution of $P2X_3$ and VR1 in a subpopulation of pulpal nerves. In this study, assessment of the peripheral subodontoblastic layer, and not the central more densely innervated area, was undertaken; this might account for any differences with previous reports.

We could not assess the relationship of the nociceptor receptors to the actual odontoblasts, as most of these were stripped from the coronal area during removal of the pulp from the chamber. It was also not possible to assess the relationship of the nociceptor immunoreactivity to the carious areas, as demineralizing of the pulp specimen rendered the immunohistochemical antibody technique invalid. In a pilot project we attempted to freeze the teeth whole and then section the teeth in order to evaluate the frozen pulp, with associated areas of caries, but this proved too difficult and the pulp fragmented.

Our finding of VR1 immunoreactive fibers in human dental pulp suggests that VR1 may play a key role in perception of heat-induced pain. Further studies are thus necessary to investigate the relationship of the intensity and chronicity of factors that regulate these receptors, which are potentially important targets for novel analgesic development.

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