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Mechanotransducers in Rat Pulpal Afferents

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Abstract

The hydrodynamic theory suggests that pain associated with stimulation of a sensitive tooth ultimately involves mechanotransduction as a consequence of fluid movement within exposed dentinal tubules. To determine whether putative mechanotransducers could underlie mechanotransduction in pulpal afferents, we used a single-cell PCR approach to screen retrogradely labeled pulpal afferents. The presence of mRNA encoding BNC-1, ASIC3, TRPV4, TRPA1, the α , β , and γ subunits of ENaC, and the two pore K⁺ channels (TREK1, TREK2) and TRAAK were screened in pulpal neurons from rats with and without pulpal inflammation. ASIC3, TRPA1, TREK1, and TREK2 were present in ~67%, 64%, 14%, and 10% of pulpal neurons, respectively. There was no detectable influence of inflammation on the proportion of neurons expressing these mechanotransducers. Given that the majority of pulpal afferents express ASIC3 and TRPA1, our results raise the possibility that these channels may be novel targets for the treatment of dentin sensitivity.

Keywords

dentin hypersensitivity; single-cell PCR; retrograde labeling; primary afferent

INTRODUCTION

While numerous mechanisms for tooth sensitivity have been proposed, the hydrodynamic theory (Brännström, 1986) is the most widely accepted. According to this theory, tooth sensitivity is the result of exposed dentin with patent dentinal tubules. When subjected to several stimuli, fluid shifts in these tubules activate sensory nerve endings at the base of the tubule and superficial pulp tissue (Andrew and Matthews, 2000). Thus, a mechanical stimulus (fluid movement) activates specific classes of nerve fibers (Dong *et al.*, 1985), resulting in the perception of pain in response to stimulation of a sensitive tooth (Jyväsjärvi and Kniffki, 1987).

Several sites for this mechanical transduction have been proposed (Pashley, 1990). One suggestion is that transduction occurs in odontoblasts present at the base of dentin tubules, which then initiate activity in pulpal afferents. Although intact odontoblasts do not appear to be critical for the generation of afferent activity in response to dentin stimulation (Hirvonen and Närhi, 1986), the presence of certain ion channels and active electrophysiological

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properties suggests a role for these cells in sensory transduction. Odontoblasts possess cation- and anion-selective channels (Guo and Davidson, 1998), and a voltage-sensitive sodium channel (Allard *et al.*, 2006) that appears to be present in the part of the odontoblast that is adjacent to nerve fibers, and a stretch-activated potassium channel (Allard *et al.*, 2000). An alternate possibility is that fluid movement within the dentin tubules directly activates afferent fibers. One prediction of this latter possibility is that messenger ribonucleic acid (mRNA) encoding the responsible mechanotransducer(s) should be present in pulpal afferents. The present study constitutes an effort to explore this possibility with a single-cell polymerase chain-reaction (PCR)-based approach used to screen for the presence of mRNA encoding known mechanotransducers in trigeminal ganglion neurons giving rise to the innervation of rat maxillary molars.

MATERIALS & METHODS

Male rats (each weighing from 150 to 250 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA) and housed in groups of 2 or 3 on a 12-12 light-dark cycle with food and water available *ad lib*. All procedures were approved by the Universities of Maryland and Pittsburgh Institutional Animal Care and Use Committees and were in accordance with the International Association for the Study of Pain guidelines for the care and use of laboratory animals.

Rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA) and rat cocktail (1 mL/kg of 55 mg/kg ketamine [Fort Dodge Animal Health, Fort Dodge, WI, USA], 5.5 mg/kg xylazine [Phoenix Scientific Inc., St. Joseph, MO, USA], and 1.1 mg/kg acepromazine [Phoenix Scientific]). Preparation of rat molars was similar to the procedure described previously (Eckert *et al.*, 1998). Occlusal cavities were prepared in second and third maxillary molars, to the dentin-pulp border. A small crystal of the retrograde tracer DiI (1,1'-di-octadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate; Invitrogen, Carlsbad, CA, USA) was placed in each cavity. The dentin cavities were brushed with self-etch primer, filled with Transbond composite (3M Unitek, Monrovia, CA, USA), and light-cured. Rats ambulated, groomed, and fed normally following recovery from anesthesia.

Tooth sensitivity is exacerbated by inflammation (Ngassapa *et al.*, 1992). To assess the possibility that this sensitization reflects an up-regulation of proteins responsible for mechanotransduction, we assessed the distribution of mechanotransducers in pulpal neurons 3 days after induction of pulpal inflammation. Pulpal inflammation was induced via the re-exposure of deep dentin (Byers, 1994).

We used a modified Evans Blue assay (Carr and Wilhelm, 1964) to assess inflammation. Following deep anesthesia, Evans Blue dye (50 mg/kg, IV, Sigma, St. Louis, MO, USA) was injected into the animals 10 min prior to their death. After trigeminal ganglia were collected, left and right alveolar ridges surrounding and including maxillary molars were sectioned and removed. Sections were incubated for 24-48 hrs in DMSO (Sigma) to extract Evans Blue. Extracted Evans Blue was quantified with a spectrophotometer (Unico, Dayton, NJ, USA) at 620 nm.

Isolated trigeminal ganglia neurons were obtained 14-17 days after pulpal labeling as previously described (Flake *et al.*, 2005). Dissociated ganglia were plated onto glass coverslips coated with 5 µg/mL mouse laminin (Gibco BRL, Carlsbad, CA, USA) and 0.1 mg/mL poly-L-ornithine (Sigma). Neurons were studied between 3 and 8 hrs after removal from the animal.

Following identification of pulpal neurons under epifluorescence illumination, neurons were collected with large-bore (~30 µm) glass pipettes (WPI, Sarasota, FL, USA) and expelled into microcentrifuge tubes containing reverse-transcriptase (RT) mix (Nealen *et al.*, 2003). RT-

PCR was performed as described elsewhere (Nealen *et al.*, 2003), with an anchored primer (5'-ttttttttttttttvn-3'; v = a, c, or g; n = a, c, g, or t [IDT, Coralville, IA, USA]) for the RT reaction and a nested PCR (Thermo-Fisher, Pittsburgh, PA, USA) amplification strategy for the PCR reaction (Nealen *et al.*, 2003). (See Appendix Table for details.)

RESULTS

The labeling procedure produced no detectable increase in Evans Blue compared with untreated controls (no cavity preparation), provided that fillings remained intact following the procedure (Fig. 1). However, re-exposure of the cavity used for labeling, 3 days prior to the animals' death, resulted in a marked increase in Evans Blue in the maxillary tissue that included the molar teeth (Fig. 1). This increase in Evans Blue was restricted to the site of repeated cavity preparation, as indicated by the results of several within-animal experiments where cavities were exposed on one side only; in these rats, the Evans Blue on the exposed side was similar to that observed in inflamed animals, while that on the intact side was similar to that of controls (data not shown).

We synthesized cDNA from mRNA extracted from whole trigeminal ganglia to screen for the presence of putative mechanotransducers: BNaC1, ASIC3, TREK1, TREK2, TRAAK, TRPA1, TRPV4, and the ENaC α , β , and γ subunits of ENaC. While BNaC1 was detected in undiluted cDNA from whole ganglia, it was undetectable following dilution of the cDNA, suggesting that channel expression would be below the level of detection at the single-cell level. The remaining ion channels and subunits were detectable at a 1:10,000 dilution of cDNA from whole ganglia (Fig. 2). The presence of these channels was assessed at the single-cell level.

Seventy-six neurons from 5 rats were collected and analyzed with respect to expression of all mechanotransducers except TRPA1 and TREK2. An additional 96 neurons from 4 rats were collected and analyzed for TREK2, and an additional 36 neurons from 3 rats were collected and analyzed for the presence of TRPA1 and ASIC3. Cyclophilin, a general housekeeping gene used to assess recovery of single cells, was detectable in the vast majority of these (70 of 76 in the first set, 84 of 96 in the second set, and 33 of 36 of the third set), indicating high efficiency in successful cell collection and cDNA synthesis. Of the cyclophilin-positive neurons, ASIC3 and TRPA1 were detected in 64% (65/102) and 63% (20/32) of pulpal neurons, respectively (Fig. 2). The majority of TRPA1-positive neurons (17 of 20) were ASIC3-positive (Fig. 2). TREK1 was detected in 15.7% (11/70) of pulpal neurons. The majority of TREK1-positive neurons (9 of 11) were also ASIC3-positive. TREK2 was detectable in 12% (10/84) of the pulpal neurons tested. Expression of other channels was either negligible (*i.e.*, ENaC α and ENaC γ) or undetectable (TRAAK and TRPV4). To control for between-animal variability, we analyzed data with the number of rats as the "n", using the percentage of neurons expressing a given channel as the statistic or measure for each animal (Fig. 3). Finally, the presence of inflammation did not appear to influence the pattern of expression of putative mechanotransducers. Labeled neurons from maxilla where the molars were drilled twice and the cavities left open demonstrated the same distribution of mechanoreceptors as labeled neurons from maxilla with molars that had a single cavity preparation that was sealed until the end of the experiment. For example, ASIC3 was present in 40 of 62 pulpal neurons from control rats and 25 of 40 neurons from inflamed rats ($p > 0.05$, Chi-square test). The proportions of neurons expressing various transducers were similar, regardless of whether the teeth had inflammation as indicated by elevated Evans blue content.

DISCUSSION

The primary observations in this study included: (1) ASIC3 is a putative mechanotransducer expressed by the majority of pulpal neurons; (2) at least 2 other putative mechanotransducers may be present in a single pulpal neuron; and (3) inflammation evoked by recent cavity preparation does not increase the percent of pulpal neurons expressing these mechanosensitive channels.

We (Behnia *et al.*, 2003) and others (Eckert *et al.*, 1998) have used DiI in the past to label pulpal neurons selectively. It is possible that dye spread outside the tooth, and that some of the neurons considered pulpal in the present study actually innervated surrounding tissue. We believe, however, that any contamination of non-pulpal afferents in the present study was low, for the following reasons. First, visual inspection of labeled teeth and surrounding tissue indicated that dye was confined to the tooth. Second, we have previously used double-labeling to confirm that there is little or no spread of dye from teeth to surrounding tissue (Behnia *et al.*, 2003). Third, we previously assessed the presence of DiI labeling in the mesencephalic nucleus V following the tooth-labeling procedure used in this study, and failed to detect any labeled neurons (Behnia *et al.*, 2003). Given that many periodontal afferents have cell bodies in the mesencephalic nucleus V, the absence of DiI in this nucleus after pulpal labeling suggests that there is little spread of the dye to the periodontal ligament.

Trigeminal ganglion neurons were dissociated and acutely cultured (3-8 hrs), to facilitate the assessment of mechanoreceptor expression in single retrogradely labeled pulpal neurons. This approach has two distinct advantages over laser-capture microdissection, which can also be used to analyze mRNA expression patterns in "single cells" (Hughes *et al.*, 2007). First, while a single-cell analysis is possible with dissociated neurons, "single cells" obtained by laser-capture cannot be completely isolated, since even thin tissue sections will contain cells above and/or below the cell of interest. Second, while not utilized in the present study, the physiological characterization of dissociated sensory neurons (Nealen *et al.*, 2003) is not possible with laser-capture approaches. Nevertheless, dissociating sensory neurons necessarily involves tissue damage, which may affect gene expression, even in acute culture (Dussor *et al.*, 2003).

The Evans Blue assay was developed as a means to measure plasma extravasation (Carr and Wilhelm, 1964), based on the amount of dye that has leaked into the tissue. However, because of the vasodilatation and increased vascular permeability associated with inflammation, and the fact that the structure of the tooth prevents swelling and therefore limits plasma extravasation, we reasoned that the total amount of dye in the jaw section (tissue plus vasculature) could be used to quantify pulpal inflammation. Consistent with this suggestion, we were able to detect a significant increase in dye content in the presence of re-exposed cavities. Importantly, this increase was evident within an animal by comparison of tissues with exposed *vs.* filled teeth. Thus, the modified Evans Blue assay appears to be an easy and sensitive method with which to quantify inflammation in teeth.

We did not observe any difference in the expression of mechanosensitive ion channels in pulpal neurons innervating teeth with inflammation compared with uninflamed teeth. In contrast, Pan and co-workers (Pan *et al.*, 2003) demonstrated a marked increase in brain-derived neurotrophic factor and a modest increase in calcitonin gene-related peptide expression following pulp exposure in fluoro-gold-labeled rat pulpal afferents. It appears that the mechanosensitive channels in pulpal neurons are constitutively expressed, unlike certain transmitters and neurotrophic factors that, to various extents, display inducible expression. The observation that mechanosensitive ion channels on pulpal neurons are present in the absence of inflammation agrees with physiological evidence that shows that intradental A-fibers

respond to stimulation of freshly exposed dentin (Hirvonen *et al.*, 1984), as well as the common clinical observation that exposed dentin in healthy teeth, especially teeth with chipped incisal edges, can be extremely sensitive. A heightened sensitivity of the intradental nerves to hydrodynamic stimuli can be demonstrated following the application of certain inflammatory mediators to exposed dentin (Ngassapa *et al.*, 1992). This sensitization may be due not to an increase in the number of mechanosensitive cells *per se*, but rather to a modulation of their functional properties, leading to enhanced responses.

The presence of TRPA1 mRNA in a subpopulation of pulpal neurons is consistent with electrophysiological and anatomical evidence, indicating that TRPA1 agonist, mustard oil, activates a subpopulation of pulpal afferents (Sunakawa *et al.*, 1999; Park *et al.*, 2001). While it is possible that this TRP channel contributes to mechanosensitivity (Corey *et al.*, 2004; Kindt *et al.*, 2007), there is little direct evidence of a role for this channel in mechanotransduction (Kwan *et al.*, 2006; Drew *et al.*, 2007). In fact, there is evidence that the channel plays no detectable role in mechanotransduction (Bautista *et al.*, 2006). We therefore suggest that the channel is more likely to function as a chemoreceptor (Bautista *et al.*, 2006) than as a mechanotransducer in pulpal neurons.

Preliminary electrophysiological analysis of dissociated pulpal neurons suggests that mechanical stimulation results in an increase in membrane conductance associated with the activation of a current with a reversal potential $\sim +40$ mV (data not shown). These results are consistent with the activation of a sodium-selective ion channel. This observation, together with our single-cell PCR results, suggests that ASIC3 may play a major role in mechanotransduction in pulpal afferents. These two observations also argue against a significant role for a potassium channel in mechanotransduction in these neurons, even though TREK1 and TREK2 were detectable in a small but significant number of pulpal neurons. They also argue against a role for mechanosensitive TRP channels, such as TRPA1 and TRPV4, which should have a reversal potential closer to 0 mV (Christensen and Corey, 2007). Interestingly, the ionic selectivity of ASIC3 suggests a mechanism to explain the desensitizing effects of high potassium levels (Markowitz *et al.*, 1991). Since this channel is relatively impermeable to potassium, increasing potassium in the extracellular space should effectively block this channel, thereby preventing neural activity.

Additional experiments would further substantiate a role for ASIC3 in mechanotransduction in pulpal afferents. First, because ASIC3 is sensitive to amiloride, this drug should block mechanically activated current in pulpal neurons. Second, because ASIC3 is also activated by protons, pulpal afferents should also be sensitive to decreases in pH. Finally, to rule out the involvement of a Ca^{2+} -selective channel, ion substitution experiments would confirm that Na^+ is the primary permeant ion species.

The prevalence of the putative mechanotransducer ASIC3 among pulpal afferents suggests that this channel may be a viable target for the design and development of novel compounds for use in the treatment of pulpal sensitivity and possibly other forms of pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A modified Evans Blue Assay may be used to assess pulpal inflammation. Evans Blue was injected systemically as described in MATERIALS & METHODS. Ten min later, tissue was harvested, and Evans Blue was extracted and quantified with spectrophotometry. Naïve animals had intact teeth. Uninflamed animals received cavities in the 2nd and 3rd molars for labeling of pulpal afferents with DiI, and then the cavities were filled with Transbond, 14-17 days prior to tissue-harvesting. Inflamed animals were treated in a manner similar to that used for uninflamed animals, except that 3 days prior to tissue harvest, cavities were re-exposed. The Evans Blue content was significantly ($p \leq 0.01$) higher in inflamed bridges than in either uninflamed or naïve sections. Numbers in parentheses are the number of animals studied in each group. Data are plotted as a mean $\pm$ standard error of the mean (SEM).

Figure 2.

The majority of the known mechanotransducers are expressed in trigeminal ganglia. **(top panel)** An ethidium-bromide-stained agarose gel loaded with products of a nested PCR amplification of cDNA generated from mRNA extracted from rat trigeminal ganglia. To assess the sensitivity of the primer pairs, we diluted the cDNA 1:10,000. We used a nested PCR strategy to increase the fidelity of the PCR reaction as well as the specificity of the product amplified. The dominant bands in lanes with multiple bands correspond to the product of the expected molecular weight. In two cases, *i.e.*, TREK1 and ENaCα, products from both the first (*i.e.*, larger PCR product) and second (*i.e.*, smaller PCR product) amplifications can be seen. **(bottom left)** Results from a PCR analysis of the contents of a single pulpal afferent collected as described in MATERIALS & METHODS. Cyclophilin was used to assess recovery of cell contents, since this is an abundant mRNA species in most cells. The only mechanotransducer detected was ASIC3. **(bottom right)** Results from a PCR analysis of the contents of 4 pulpal neurons collected as described in MATERIALS & METHODS. cDNA generated from each afferent was probed for the presence of TRPA1 and ASIC3. Both genes were detected in the 1st and 4th afferents, while only one or the other was detected in the 2nd and 3rd neurons. In all three panels, lanes labeled BP were loaded with a DNA ladder used to estimate the size of PCR products.

Figure 3.

Frequency distribution of known mechanotransducers among pulpal neurons. Each bar represents the mean ($\pm$ SEM) proportion of pulpal neurons observed in each rat. $N = 5$ for all except ASIC3, where $n = 8$, TRPA1, where $n = 3$, and TREK2, where $n = 4$. ASIC3 and TRPA1 were present in pulpal neurons at a higher frequency ($p < 0.01$) than any other transducer assessed.