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Research report

Expression of transient receptor potential vanilloid 1 and anoctamin 1 in rat trigeminal ganglion neurons innervating the tongue

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ABSTRACT

Transient receptor potential vanilloid 1 (TRPV1) is a polymodal sensor that is activated by heat (>43 °C), acid, or capsaicin, the pungent ingredient of hot peppers. Reports that mice lacking TRPV1 display heat avoidance behaviors and TRPV1-negative neurons respond to heat suggest that an additional heat sensor is present. Anoctamin 1 (ANO1; also known as transmembrane protein 16A [TMEM16A]), is a component of Ca^{2+}-activated chloride channels (CaCCs), and has been recently identified as a heat sensor, activated by temperatures over 44 °C. ANO1 is highly co-localized with TRPV1 in small-diameter dorsal root ganglion (DRG) neurons. The aim of the present study was to investigate co-expression of ANO1 and TRPV1 in rat trigeminal ganglion (TG) neurons innervating the tongue by using retrograde labeling and immunohistochemical techniques. Fluoro-gold (FG) retrograde labeling was used to identify the TG neurons innervating the anterior two thirds of the tongue; as expected, most labeling was detected in the mandibular division of the TGs. The FG-labeled TG neurons showed TRPV1 immunoreactivity (17.9%) and ANO1 immunoreactivity (13.7%), indicating that TRPV1- and ANO1-expressing neurons were present in the mandibular division of the TGs. Seventy-six percent of the ANO1-immunoreactive TG neurons were also immunoreactive for TRPV1; this co-expression was mainly detected in small- to medium-diameter TG neurons. The high degree of co-expression of TRPV1 and ANO1 suggests that cooperation between ANO1 and TRPV1 plays a role in the signaling pathways of nociceptive TG neurons.

1. Introduction

Transient receptor potential vanilloid 1 (TRPV1) is polymodal sensor that is activated by heat (>43 °C), acid, or capsaicin, the pungent ingredient of hot peppers (Caterina and Julius, 2001; Caterina et al., 1997; Tominaga, 2008; Tominaga et al., 1998; Tominaga and Julius, 2000). *Trpv1*$^{-/-}$ mice show reduced thermal hyperalgesia and inflammatory pain (Caterina et al., 2000; Davis et al., 2000); however, heat avoidance behaviors remain in mice lacking TRPV1 and TRPV1-negative neurons respond to heat (Basbaum et al., 2009; Davis et al., 2000; Woodbury et al., 2004), suggesting the presence of an additional heat sensor. Anoctamin 1 (ANO1), which is also known as transmembrane protein 16A (TMEM16A), a component of Ca^{2+}-activated chloride channels (CaCCs), possesses eight transmembrane domains and conducts chloride currents activated by intracellular Ca^{2+} (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Recently, Cho et al. (2012) reported the following findings: (1) ANO1 is expressed in small-diameter dorsal root ganglion (DRG) neurons and is highly co-localized with TRPV1; (2) ANO1 is activated by temperatures greater than 44 °C; (3) heat-induced depolarization in mouse *Trpv1*$^{-/-}$ DRG neurons is blocked by an antagonist of endogenous CaCCs; (4) knockdown or deletion of ANO1 in DRG neurons substantially reduces nociceptive behavior in thermal pain models. These findings strongly suggest that ANO1 is a heat sensor that detects nociceptive thermal stimuli in DRG sensory neurons and that it possibly mediates nociception.

Although ANO1 has not been detected in rat nasal trigeminal sensory fibers by immunohistochemistry (Dauner et al., 2012), ANO1 transcripts have been detected in mouse trigeminal ganglia (TGs) (Schöbel et al., 2012). Schöbel et al. (2012) found that pharmacological inhibition of CaCCs reduces the amplitude of capsaicin-induced responses of trigeminal ganglion (TG) neurons. TRPV1 immunoreactivity is detected in approximately 20% of TG neurons; these TRPV1-positive neurons are mostly small- to medium-sized (Ichikawa and Sugimoto, 2001).

Abbreviations: ANO1, anoctamin 1; BK, bradykinin; BMS, burning mouth syndrome; CaCCs, Ca^{2+}-activated chloride channels; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; FG, fluoro-gold; NKCC1, Na^+-K^+-$2Cl^-$ cotransporter 1; O.C.T. compound, Optimal Cutting Temperature compound; TG, trigeminal ganglion; TMEM16A, transmembrane protein 16A; TRPV1, transient receptor potential vanilloid 1.

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Retrogradely labeled TG neurons from the facial skin and tooth pulp exhibit TRPV1 immunoreactivity, and TRPV1 and calcitonin gene-related peptide (CGRP) are co-expressed in cutaneous neurons and tooth pulp neurons in the TG (Ichikawa and Sugimoto, 2001). Capsaicin evokes a sensation of burning pain by stimulating TRPV1 on primary afferent neurons, and TRPV1 is expressed not only in primary afferents but also in oral epithelial cells (Kido et al., 2003). Furthermore, the proportion of TRPV1-positive neurons is abnormally high in patients with burning mouth syndrome (BMS) (Yilmaz et al., 2007), which is an idiopathic and often chronic and intractable pain condition. The tongue is highly innervated and routinely comes in contact with noxious thermal, chemical, and mechanical stimuli and the anterior two thirds of the tongue is rich in afferents that convey sensory stimuli via the lingual nerve to cell bodies in the TG (Elitt et al., 2008). Trigeminal afferent neurons retrogradely labeled by the injection of wheat germ agglutinin into the tip of the tongue express TRPV1 (Elitt et al., 2008). Currently, co-expression of TRPV1 and ANO1 in TG neurons has not been addressed. Therefore, in this study, we used retrograde labeling and immunohistochemical techniques to investigate co-expression of ANO1 and TRPV1 in TG neurons innervating the anterior two thirds of the tongue.

2. Materials and methods

2.1. Animals

All experiments were approved by the Animal Use and Care Committee of Nippon Dental University and were conducted in accordance with the ethical and animal welfare issues in the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). All appropriate efforts were made to minimize the number of animals used in this study. We used 10 Wistar rat pups (aged P9–P10; 5 males, 5 females; weighing 19–22 g). Gender differences were not investigated in this study.

2.2. Retrograde labeling of TG neurons innervating the tongue

For immunohistochemical studies, neurons were first labeled with fluoro-gold (FG, Fluorochrome, Englewood, CO, USA) as follows. Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and injected with atropine sulfate (0.5 mg/kg, s.c.) (Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) to prevent suppression of respiration caused by tracheal secretion. After extracting the tongue slightly from the animal's mouth, FG solution (2% in distilled water, 20 μl) were injected bilaterally into the anterior two thirds of the tongue by using an injection needle (diameter: 0.4 mm, 27G) affixed to a 10 μl Hamilton syringe (dead space filled with liquid paraffin). To prevent the spread of the tracer to adjacent structures, cotton swabs were used after the injections. The localization of FG around the injection site in the tongue was verified. Animals were returned to a lactating mother and allowed to recover for 5 days.

2.3. Tissue preparation and immunohistochemistry of TRPV1 and ANO1

Five days after the injection, the animals were euthanized under deep anesthesia with sodium pentobarbital (200 mg/kg i.p.). The TGs were removed from the animals and immediately fixed in 4% w/v paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 1–2 h. The fixed tissues were then cryoprotected by step-wise immersion for 30 min periods in 10%, 20%, and 30% (w/v) sucrose, respectively, in 0.1 M phosphate buffer (pH 7.4) at 4 °C, and then 30% sucrose solution overnight. The TGs were embedded with Optimal Cutting Temperature (O.C.T.) compound. Frozen sections were made at a thickness of 20 μm by using a cryostat (CM3050S, Leica Microsystems GmbH, Wetzlar, Germany). To prevent recounting of neurons, every fourth section was used for analysis. Sections were mounted on glass slides (FRC-01, Matsunami Glass Ind., Ltd., Osaka, Japan), dried for 2 h at room temperature, and rinsed three times (5 min each time) in phosphate buffer saline containing 0.1% (v/v) Tween 20 (tPBS). Rinsed frozen sections were incubated in heated antigen retrieval solution (Histo VT One; Nakalai Tesque, Inc., Kyoto, Japan) for 20 min at 70 °C. Antigen-retrieved sections were rinsed three times (5 min each time) in tPBS, and then covered with sufficient blocking solution (Blocking One Histo, Nakalai Tesque, Inc.) to block non-specific binding. The sections were incubated at room temperature for 10 min and washed with tPBS for 5 min before incubation with the following primary antibodies diluted in blocking buffer diluted with tPBS (1:20) at 4 °C overnight: TRPV1 C-terminus (Capsaicin receptor) (1:100; GP14100,

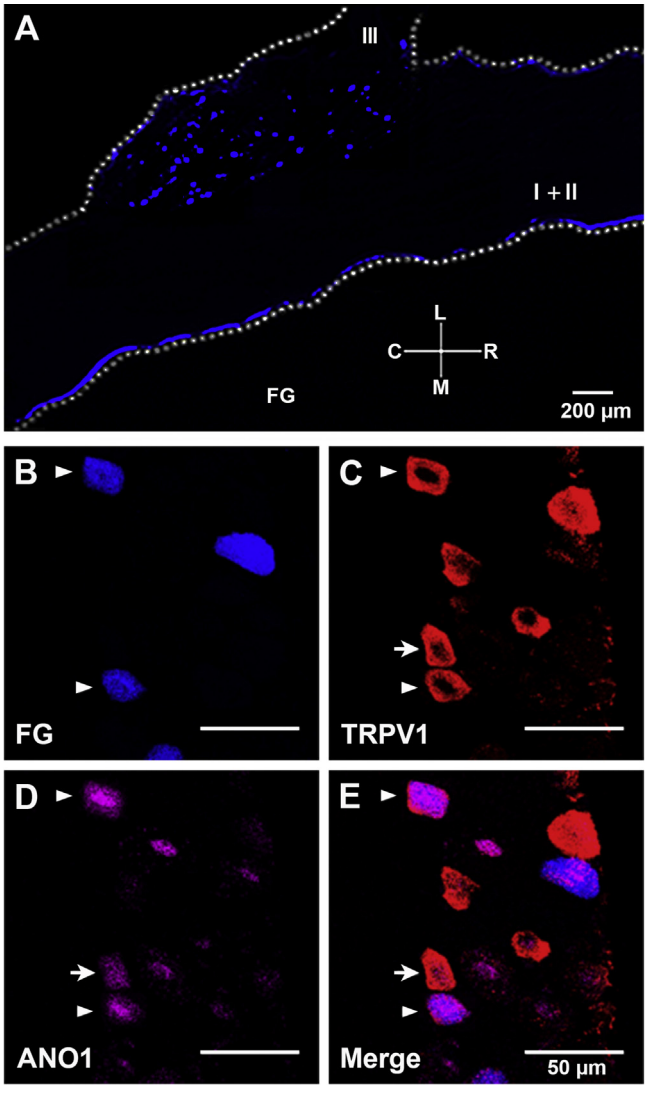

Fig. 1. Microphotographs of transient receptor potential vanilloid 1 (TRPV1) and anoctamin 1 (ANO1) immunoreactivity in fluoro-gold (FG)-labeled trigeminal ganglion (TG) neurons. (A) FG-labeled neurons located in the mandibular division of the TG. Scale bar, 200 μm. R, rostral; C, caudal; L, lateral; M, Medial. (B) FG-labeled TG neurons. TRPV1- (C) and ANO1- (D) immunoreactive TG neurons. (E) Merged image. An ANO1- and TRPV1-immunoreactive TG neuron is indicated by an arrow. (B–E) A typical example of FG-labeled TG neurons co-expressing TRPV1 and ANO1 are indicated by filled triangles. Scale bar, 50 μm.