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THE EXPRESSION OF HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNEL 1 (HCN1) AND HCN2 IN THE RAT TRIGEMINAL GANGLION, SENSORY ROOT, AND DENTAL PULP

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Abstract—Hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) and 2 (HCN2) are abundantly expressed in primary sensory neurons and contribute to neuronal excitability and pathological pain. We studied the expression of HCN1 and HCN2 in the rat trigeminal ganglion (TG) neurons and axons in the dental pulp, and the changes in their expression following inflammation, using light- and electron-microscopic immunocytochemistry and quantitative analysis. HCN1 and HCN2 were expressed predominantly in large-sized, neurofilament 200-immunopositive (+) or parvalbumin+ soma in the TG whereas they were expressed mostly in unmyelinated and small myelinated axons in the sensory root. The expression was particularly strong along the plasma membrane in the soma. In the dental pulp, majority of HCN1+ and HCN2+ axons coexpressed calcitonin gene-related peptide. They were expressed mainly in the peripheral pulp and pulp horn where the axons branch extensively in the dental pulp. The expression of HCN1 and HCN2 in TG neurons increased significantly in rats with experimentally induced inflammation of the dental pulp. Our findings support the notion that HCN1 and HCN2 are expressed mainly by both the soma of mechanosensitive neurons in the TG and peripheral axons of nociceptive neurons in the sensory root, and may play a role in the mechanisms of inflammatory pain from the dental pulp.

Key words: HCN, axon type, trigeminal ganglion, dental pulp, inflammation, ultrastructure.

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Abbreviations: CGRP, calcitonin gene-related peptide; CFA, Complete Freund's Adjuvant; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; IgG, immunoglobulin G; Ih, hyperpolarization-activated currents; LM, light microscopy; LTM, low threshold mechanoreceptive; NDS, normal donkey serum; NF200, neurofilament 200; PB, phosphate buffer; PBS, phosphate-buffered saline; PGP9.5, protein gene product 9.5; TG, trigeminal ganglion; VGLUT, vesicular glutamate transporter.

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INTRODUCTION

Hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) and 2 (HCN2), of the discovered four isoforms of HCN channels, are most abundantly expressed in the primary sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion (TG, Chaplan et al., 2003; Yao et al., 2003). They contribute to hyperpolarization-activated currents (Ih) and neuronal excitability, and are implicated in pathological pain (Chaplan et al., 2003; Tu et al., 2004; Emery et al., 2011; Weng et al., 2012). What type of neuron in the DRG and TG expresses HCN1 and HCN2 is still uncertain: the expression in large-sized soma (Chaplan et al., 2003; Hogan and Poroli, 2008; Cho et al., 2009; Acosta et al., 2012; Gao et al., 2012; Schnorr et al., 2014) suggests that HCN1- and HCN2-positive neurons are predominantly low threshold mechanoreceptive (LTM), whereas studies that implicate HCN in allodynia and hyperalgesia following inflammation and nerve injury (Chaplan et al., 2003; Cho et al., 2009; Emery et al., 2012) suggest that HCN may be expressed in small, nociceptive neurons.

Peripheral application of a specific HCN blocker attenuated mechanical hypersensitivity in a model of inflammation (Weng et al., 2012; Hatch et al., 2013), suggesting that HCN expressed in peripheral axons plays a role in the peripheral sensitization and inflammatory pain. However, little is known about the HCN expression in peripheral axons, particularly in the dental pulp. Dental pulp is innervated densely by nociceptive afferents that signal pain in response to a variety of stimuli (Byers, 1984; Hildebrand et al., 1995; Paik et al., 2009). We showed that glutamate receptors and vesicular glutamate transporters (VGLUTs) are expressed in axons of the dental pulp (Kim et al., 2009; Paik et al., 2012), suggesting peripheral pain processing in the pulp. In addition, even though HCN1 and HCN2 are implicated in inflammatory pain (Chaplan et al., 2003; Emery et al., 2012; Schnorr et al., 2014), it remains uncertain whether their expression in TG neurons is associated with pulpal inflammatory pain (Wells et al., 2007).

To address these issues, we studied the expression of HCN1 and HCN2 in the soma and axons in the rat TG neurons and axons in the dental pulp, and the changes in that expression following inflammation, using light- and electron-microscopic immunocytochemistry and quantitative analysis.

EXPERIMENTAL PROCEDURES

All animal procedures were performed according to the National Institutes of Health guidelines and were approved by the Kyungpook National University Intramural Animal Care and Use Committee. Experiments were designed to minimize the number of animals used and their suffering. Thirty male Sprague–Dawley rats weighing 300–320 g were used for this study: Fifteen rats were for light microscopic (LM) immunohistochemistry including three for normal group, and each three for Complete Freund's Adjuvant (CFA) treatment with 1-day, 3-day survival and their controls, and three rats were for electron microscopic (EM) immunohistochemistry. Twelve rats were for Western blot analysis including each three for CFA treatment with 1-day, 3-days survival and their controls.

Tooth pulp inflammation model

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and the occlusal enamel and dentin of the right maxillary 1st (M1) and 2nd (M2) molars were filed off to just before exposing the pulp using a low-speed dental drill with a round bur under water-cooling. A small piece of tissue paper soaked in 50% solution of CFA in saline or saline for control was applied to the exposed dentinal surfaces for 5 min, and after that the dentinal surfaces were sealed with dental cement. One or 3 days later, rats were re-anesthetized and sacrificed with intravascular perfusion with fixatives.

LM immunohistochemistry

For immunofluorescence, rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.), transcardially perfused with heparinized normal saline, followed by freshly prepared fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The dental pulps of the right maxillary 1st (M1) and 2nd (M2) molars and the right TGs in normal rats, and the right TGs of CFA-treated rats (experimental) and of saline-treated rats (control) were carefully removed, postfixed in the same fixative for 2 h, and cryoprotected in 30% sucrose in PB overnight at 4 °C. The next day, 30-μm-thick sections were cut on a freezing microtome and treated with 50% ethanol for 30 min and with 10% normal donkey serum (NDS, Jackson ImmunoResearch, West Groove, PA, USA) for 30 min. Sections were incubated overnight in rabbit anti-HCN1 (1:800, AB5884, Chemicon, Billerica, MA, USA) or rabbit anti-HCN2 (1:800, APC-030, Alomone, Jerusalem, Israel) antibodies alone or in combination with mouse anti-protein gene product 9.5 (PGP 9.5, 1:5,000, YM8104, Accurate chemical and scientific Corp., Westbury, NY, USA) antibodies. To verify the identity of HCN1- and HCN2-immunopositive soma and pulpal axons, sections were also incubated overnight in rabbit anti-HCN1 or rabbit anti-HCN2 antibodies in combination with mouse anti-neurofilament 200 (NF200, 1:100,000, N0142, Sigma–Aldrich, St.Louis, MO, USA), mouse anti-parvalbumin (1:1,000, MAB1572, Chemicon), mouse anti-calcitonin gene-related peptide (CGRP, 1:1,000, ab81887, Abcam, Cambridge, MA, USA) or biotinylated-isolectin B4 (biotinylated-IB4, 5 μg/ml, L-3759, Sigma–Aldrich). On the next day, the sections were washed with phosphate-buffered saline (PBS; 0.01 M, pH7.4) and incubated for 3 h with Cy3- or fluorescein isothiocyanate-conjugated secondary antibodies (raised in donkey, 1:200, in PB, Jackson ImmunoResearch). For biotinylated-IB4 staining, sections were incubated for 3 h with streptavidin-Alexa488 (1:600, S-11223, Molecular probes, Grand Island, NY, USA). Finally, sections were mounted on slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA), and micrographs were obtained with an Exi digital camera (Q-Imaging Inc., Surrey, CA, USA), attached to a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Inc., Jena, Germany).

Quantitative analysis

To assess the size distribution of HCN1-immunopositive (+) and HCN2+ neurons in the TG, cross-sectional area of all neuronal somata with clearly visible nucleoli was measured using Image J software (http://imagej.nih.gov/ij/, NIH, Bethesda, MD, USA) and graphs were built in KaleidaGraph (v 3.5; Synergy Software, Reading, PA, USA). HCN1+ and HCN2+ soma were divided into three groups, according to their cross-sectional area: small ($<600\ \mu m^2$ in cross-sectional area), medium (600–1200 μm^2) and large ($>1200\ \mu m^2$) and their proportions were analyzed.

To quantify the immunoreactivity in HCN1+ and HCN2+ TG perikarya in the CFA-treated rats, a total of 12–16 images were collected from 3 to 4 sections of each TGs in each saline- and CFA-treated rat (1-day or 3-days). The images were captured with a 20× objective (857.14 × 652.94 μm, 1360 × 1036 pixels) from the maxillary region of the TG. The images were converted to grayscale and all images for each antibody were identically enhanced for brightness and contrast. The

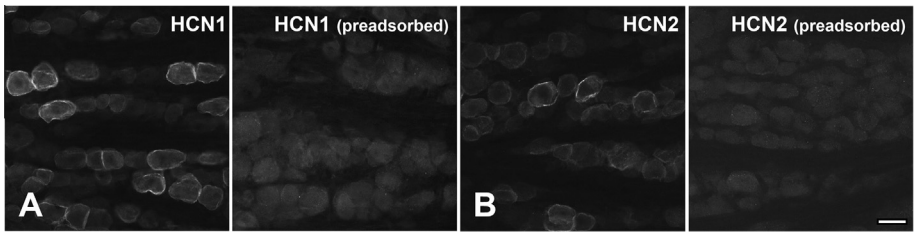

Fig. 1. Immunofluorescent staining for HCN1 (A) and HCN2 (B) in trigeminal ganglion neurons is completely abolished by preadsorption with the corresponding blocking peptides (HCN1: 1 μg/ml, HCN2: 1 μg/ml). Scale bar = 50 μm.