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THE DISTRIBUTION OF LOW-THRESHOLD TTX-RESISTANT NA$^+$ CURRENTS IN RAT TRIGEMINAL GANGLION CELLS

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Abstract—The distribution of low-threshold tetrodotoxin-resistant (TTX-r) Na$^+$ current and its co-expression with high-threshold TTX-r Na$^+$ current were studied in randomly selected acutely dissociated rat trigeminal ganglion (non-identified TG cells) and TG cells serving the temporomandibular joint (TMJ-TG cells). Conditions previously shown to enhance Na$_V$1.9 channel-mediated currents (holding potential (HP) −80 mV, 130-mM fluoride internally) were employed to amplify the low-threshold Na$^+$ current. Under these conditions, detectable low-threshold Na$^+$ current was exhibited by 16 out of 21 non-identified TG cells (average, 1810 ± 358 pA), and by nine of 14 TMJ-TG cells (average, 959 ± 525 pA). The low-threshold Na$^+$ current began to activate around −55 mV and was inactivated by holding TG cells at −60 mV and delivering 40-ms test potentials (TPs) to 0 mV. The inactivation was long lasting, recovering only 8 ± 3% over a 5-min period after the HP was returned to −80 mV. Following low-threshold Na$^+$ current inactivation, high-threshold TTX-r Na$^+$ current, evoked from HP −60 mV, was observed. High-threshold Na$^+$ current amplitude averaged 16,592 ± 3913 pA for TPs to 0 mV, was first detectable at an average TP of −34 ± 1.3 mV, and was ½ activated at −7.1 ± 2.3 mV. In TG cells expressing prominent low-threshold Na$^+$ currents, changing the external solution to one containing 0 mM Na$^+$ reduced the amount of current required to hold the cells at −80 mV through −50 mV, the peak effect being observed at HP −60 mV. TG cells recorded from with a more physiological pipette solution containing chloride instead of fluoride exhibited small low-threshold Na$^+$ currents, which were greatly increased upon superfusion of the TG cells with the adenylyl cyclase (AC) activator forskolin. These data suggest two hypotheses: (1) low- and high-threshold Na$_V$1.9 and Na$_V$1.8 channels, respectively, are frequently co-expressed in TG neurons serving the TMJ and other structures, and (2), Na$_V$1.9 channel-mediated currents are small under physiological conditions, but may be enhanced by inflammatory mediators that increase AC activity, and may mediate an inward leak that depolarizes TG neurons, increasing their excitability. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Na$_V$1.9, trigeminal ganglion, temporomandibular joint, sensory neuron, hyperalgesia, inflammation.

INTRODUCTION

Na$_V$1.9 channels mediate a low-threshold tetrodotoxin-resistant (TTX-r) Na$^+$ current, which begins to activate at significantly more negative voltages than the TTX-r Na$_V$1.8 channels that mediate action potentials in nociceptor somata. Previous studies indicate that Na$_V$1.9 channels are expressed on somata, axons, peripheral receptors, and central terminals in a large proportion of nociceptors, suggesting they play an important role in nociceptor function (Amaya et al., 2000; Black and Waxman, 2002; Fang et al., 2002; Coggeshall et al., 2004; Padilla et al., 2007; Wells et al., 2007; Keh et al., 2008). Electrophysiology experiments on isolated dorsal root ganglion (DRG) neurons and modeling studies have led to the hypothesis that Na$_V$1.9 channels are involved in the modulation of nociceptor membrane potential and excitability, and may enhance various types of firing behavior such as burst firing and rebound firing (Herzog et al., 2001; Baker et al., 2003; Baker, 2005; Maingret et al., 2008; Ostman et al., 2008). Additional studies suggest that Na$_V$1.9 channels play an important role in the transmission of inflammatory pain. Na$_V$1.9 channel expression was up-regulated in the cell bodies of afferents serving rat knee joint, following injection of carrageenan or Freund's adjuvant into the joint, and in nociceptors serving inflamed tooth pulp in humans (Wells et al., 2007; Strickland et al., 2008; Warren et al., 2008). Also, Na$_V$1.9 channel knock-out mice exhibited deficits in mechanical and/or thermal hyperalgesia following injection of carrageenan, formalin, Freund's adjuvant, or the inflammatory mediators bradykinin and prostaglandin E_2 (Priest et al., 2005; Amaya et al., 2006).

Although Na$_V$1.9 channels have been observed in trigeminal ganglion (TG) cell bodies using immunohistochemical techniques (Dib-Hajj et al., 1998, 2002; Wells et al., 2007), there are few, if any, reports of Na$_V$1.9-mediated currents recorded directly from these neurons using electrophysiological techniques. The purpose of the present study was to examine rat TG cells, including those serving the temporomandibular

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Abbreviations: AC, adenylyl cyclase; ACSF, artificial cerebral spinal fluid; A-D/D-A, analog to digital/digital to analog; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; GTP, guanosine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HP, holding potential; MΩ, megaohm; PKA, protein kinase A; PKC, protein kinase C; TEA, tetraethylammonium chloride; TEA-OH, tetraethylammonium hydroxide; TG, trigeminal ganglion; TMJ, temporomandibular joint; TP, test potential; TTX, tetrodotoxin.

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joint (TMJ), regarding their expression and modulation of TTX-resistant low-threshold Na^+ currents, which could be mediated by $Na_V1.9$ channels, using techniques previously shown to minimize inactivation of, and amplify $Na_V1.9$ channel-mediated currents.

The TMJ appeared to be a good candidate for a specific structure that might be innervated by $Na_V1.9$ channel-expressing TG afferents, since a previous study (mentioned above) demonstrated $Na_V1.9$ channel expression by afferents serving the rat knee joint. Furthermore, many people worldwide suffer from TMJ disorder, which could involve $Na_V1.9$ channel-mediated inflammatory pain. Thus, TG afferents specifically serving the TMJ were identified by 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide (DiI) labeling and explored regarding their possible expression of $Na_V1.9$ channels.

EXPERIMENTAL PROCEDURES

Preparation of TG cells

All procedures involving animals were approved by the University of Tennessee Health Science Animal Use and Care Committee. Adult male and female rats (≈250 g), Sprague–Dawley (purchased from Harlan Indianapolis, IN, USA) were rendered unconscious with isoflurane or ketamine/xylazine, decapitated, and the lateral portion of the TG containing predominately cell bodies associated with the maxillary division was dissected out. The fragments of TG were maintained up to 6 h in artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.24 KH2PO4, 26 NaHCO3, 1.3 MgCl2, 2.4 CaCl2, 10 glucose, and continuously bubbled with 5% CO2/95% O2. Before recordings, TG fragments were incubated at 34 °C for 1/2 h in ACSF containing 2 mg/ml collagenase type 1A (Sigma–Aldrich St. Louis, MO, USA) and 2 mg/ml protease (Sigma–Aldrich). The enzymes were washed away from the TG fragments with Tyrodes' solution containing (in mM): 140 NaCl, 4 KCl, 2 MgCl2, 2 CaCl2, 10 glucose, 10 HEPES, pH 7.4 with NaOH. Individual TG cell bodies were dispersed by trituration in Tyrodes' solution. A suspension of isolated TG cells was immediately transferred to a plastic 35-mm petri dish where many adhered to the bottom within 5 min, and were then superfused with Tyrodes' solution at a rate of around 2 ml per minute.

DiI labeling of temporomandibular joint afferents

In some cases the rats were pretreated with an injection of DiI (Molecular Probes, Eugene, OR, USA) into the TMJ approximately 1 week before TG were harvested. DiI for injection was dissolved at a concentration of 8.5 mg/ml in dimethyl sulfoxide (DMSO) and sterilized by filtration. Prior to injection, rats were deeply anesthetized with isoflurane, continuously applied with an anesthesia machine at a concentration of 4–5% through a small mask that covered the rat's nose. The area surrounding the TMJ was shaved and disinfected with betadiene. The tip of the needle (30 gauge, attached to a Hamilton syringe) was directed through skin and muscle just below the zygomatic arch, perpendicular to the ramus of the mandible, until contact with the ramus was made. Then the needle was directed superiorly into the TMJ capsule. After slowly injecting 3 μl of DiI solution, the needle was left in place for about a minute to reduce fluid seepage out of the TMJ (Flake and Gold, 2005). Rats were then taken off anesthesia and monitored for several hours after awakening. Rats showed no signs of pain or difficulty drinking and eating, and there were no obvious signs of inflammation at the injection site at any time between the injection and TG harvesting. At the time when TG were harvested, the area surrounding the TMJ was dissected and examined for the presence of DiI. Then the TMJ capsule was opened and inspected for the presence of DiI. Tissues injected with DiI are stained with its characteristic purple color, and are easily recognized. Only cells isolated from rats where DiI was found within the TMJ, with minimal contamination of surrounding tissues are included in the data. During electrophysiology experiments, DiI-labeled TG cell bodies were detected using epifluorescence.

Whole-cell patch clamp recording

Whole-cell recordings were carried out using 35-mm petri dishes as baths. Data acquisition and analysis were accomplished with P-Clamp 8.2, using an Axopatch 200A amplifier coupled with a Digidata A-D/D-A converter (Molecular Devices, Sunnyvale, CA, USA). Data were leak subtracted using the P/4 method. Patch electrodes were coated with Sylgard to about 200 μm from the tip, and fire polished to a final resistance of 0.8–2.0 M. In most experiments, the internal solution contained (in mM); 130 CsF, 1 $MgCl_2$, 10 NaCl, 11 EGTA, 10 HEPES, pH 7.4 with CsOH. In experiments testing channel modulation with forskolin, the internal solution contained (in mM) 120 CsCl, 5 2Na-ATP, 0.4 2Li-GTP, 5 $MgCl_2$, 5 EGTA, 1.86 $CaCl_2$, 10 HEPES, adjusted to pH 7.4 with CsOH. (Total $[Ca^{2+}]_i$ is calculated to be 100 nM.)

At the beginning of each recording, cell diameter was measured using a graticule in the microscope objective, and is reported as the average of the shortest and longest distances (at right angles to each other) across the middle of the cell. While the cells were being superfused with Tyrodes' solution, a simple voltage protocol was delivered to test for various patterns of current expression (current signatures) that had been used previously to categorize DRG cells (Cardenas et al., 1995; see Fig. 3A, B, D). Following this, TTX-resistant Na^+ currents were recorded in an external solution containing (in mM): 140 NaCl, 10 TEA, 2 $CaCl_2$, 5 $MgCl_2$, 10 HEPES, 0.1 $CdCl_2$, 0.0005 TTX, pH 7.4 with TEA-OH (final concentration of TEA ≈ 14 mM). TTX-r Na^+ currents were eliminated using an external solution identical to the above, except 140 mM NaCl was replaced with 140 mM choline-Cl. Solutions were changed around the cell under study by means of a small glass capillary tube placed near the cell in the bath, as described earlier (Cardenas et al., 1997). Chemicals and salts used to make up the external and pipette solutions were obtained from Sigma–Aldrich. The patch clamp amplifier was tuned to null the whole-cell capacity transients, prediction was typically set at 80–85%, and series resistance was compensated by 80–85%. To calculate the final uncompensated series resistance after tuning the amplifier, whole-cell capacity transients were recorded before amplifier tuning. These transients were used to estimate the series resistance before compensation so that the series resistance remaining after compensation could be calculated. The estimates of uncompensated series resistance were used to correct plots of conductance versus test potential (TP) that were used to estimate the voltage at which high-threshold TTX-r Na^+ currents were ½ activated (see Tripathi et al., 2006 for details). Plots of low- and high-threshold Na^+ current amplitude versus TP were also adjusted for uncompensated series resistance.

Statistical analysis

All statistical analyses were preformed in Systat (SPSS, Inc. Chicago, IL, USA). The significance of differences in the means of paired data or independent data was determined using the

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