Neuroscience 270 (2014) 192–202

N-TYPE CALCIUM CURRENT, CAV2.2, IS ENHANCED IN SMALL-DIAMETER SENSORY NEURONS ISOLATED FROM *NF1*+/– MICE

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Abstract—Major aspects of neuronal function are regulated by Ca^{2+} including neurotransmitter release, excitability, developmental plasticity, and gene expression. We reported previously that sensory neurons isolated from a mouse model with a heterozygous mutation of the *Nf1* gene (*Nf1*+/–) exhibited both greater excitability and evoked release of neuropeptides compared to wildtype mice. Furthermore, augmented voltage-dependent sodium currents but not potassium currents contribute to the enhanced excitability. To determine the mechanisms giving rise to the enhanced release of substance P and calcitonin gene-related peptide in the *Nf1*+/– sensory neurons, the potential differences in the total voltage-dependent calcium current (I_{Ca}) as well as the contributions of individual Ca^{2+} channel subtypes were assessed. Whole-cell patch-clamp recordings from small-diameter capsaicin-sensitive sensory neurons demonstrated that the average peak I_{Ca} densities were not different between the two genotypes. However, by using selective blockers of channel subtypes, the current density of N-type (Cav2.2) I_{Ca} was significantly larger in *Nf1*+/– neurons compared to wildtype neurons. In contrast, there were no significant differences in L-, P/Q- and R-type currents between the two genotypes. Quantitative real-time polymerase chain reaction measurements made from the isolated but intact dorsal root ganglia indicated that N-type (Cav2.2) and P/Q-type (Cav2.1) Ca^{2+} channels exhibited the highest mRNA expression levels although there were no significant differences in the levels of mRNA expression between the genotypes. These results suggest that the augmented N-type (Cav2.2) I_{Ca} observed in the *Nf1*+/– sensory neurons does not result from genomic differences but may reflect post-translational or some other non-genomic modifications. Thus, our results demonstrate that sensory neurons from *Nf1*+/– mice, exhibit increased N-type I_{Ca} and likely account for the increased release of substance P and calcitonin gene-related peptide that occurs in *Nf1*+/– sensory neurons.

Key words: neurofibromatosis, calcium channels, dorsal root ganglia, qPCR, mRNA.

INTRODUCTION

Neurofibromatosis type 1 (Nf1) is a common genetic disorder characterized by tumor formation. People with Nf1 also experience a more intense painful response to stimuli than do unaffected individuals. It is likely that these abnormal painful states involve the sensitization of small-diameter nociceptive sensory neurons that are known to mediate the transmission of pain and itch. Previously, we demonstrated that small-diameter capsaicin-sensitive sensory neurons isolated from mice with a heterozygous mutation of the *Nf1* gene (coding for the protein neurofibromin) have augmented excitability compared to wildtype neurons (Wang et al., 2005). Consistent with this enhanced excitability, the peak current densities for both tetrodotoxin-sensitive and -resistant sodium currents (Wang et al., 2010a) as well as the expression of mRNA for specific sodium channel subtypes (Hodgdon et al., 2012) were significantly larger in *Nf1*+/– sensory neurons. However, neither delayed rectifier nor A-type potassium currents were altered in *Nf1*+/– neurons (Wang et al., 2010a). Furthermore, stimulus-evoked release of the neuropeptides, substance P and calcitonin gene-related peptide (CGRP), was significantly higher from sensory neurons isolated from *Nf1*+/– mice (Hingtgen et al., 2006). Therefore, it is reasonable to speculate that the calcium currents and/or expression of these channels is higher in adult sensory neurons with heterozygous mutation of the Nf1 gene (*Nf1*+/–) than that of wildtype cells.

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Abbreviations: Arbp, acidic ribosomal protein P0; CGRP, calcitonin gene-related peptide; Cq, quantification cycle; DRG, dorsal root ganglia; E_{Ca}, reversal potential for I_{Ca}; EGTA, ethylenediamine tetraacetic acid; *G*, conductance; G_{max}, maximal conductance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPRT, hypoxanthine–guanine phosphoribosyl transferase; I_{Ca}, Ca^{2+} current; *k*, slope factor; MPL, 1-methyl-2-pyrrolidinone; Nf1, neurofibromatosis type 1; qPCR, real-time quantitative polymerase chain reaction; TEA, tetraethyl ammonium; $V_{0.5}$, voltage for half-maximal activation; V_m, membrane potential; ω-AgTx, ω-agatoxin IVA; ω-CTx, ω-conotoxin GVIA.

http://dx.doi.org/10.1016/j.neuroscience.2014.04.021

Multiple experimental approaches established that voltage-dependent Ca^{2+} channels are important for neurotransmitter release (Catterall, 2000; Catterall et al., 2005; Catterall and Few, 2008; Neher and Sakaba, 2008; Atlas, 2013) and nociceptive neurotransmission (Malmberg and Yaksh, 1994; Kim et al., 2001; Saegusa et al., 2001; Winquist et al., 2005; Cregg et al., 2010; Todorovic and Jevtovic-Todorovic, 2011; Lipscombe et al., 2013). Based on molecular, biophysical, and pharmacological properties, high-threshold voltage-activated Ca^{2+} channels have been classified into L, N, P/Q and R subtypes (Fox et al., 1987; Diochot et al., 1995; Jones, 1998; Triggle, 1999; Dolphin, 2009). The variety of Ca^{2+} channels found in neuronal membranes suggests that each type plays a distinct physiological role. For instance, L- and N-type currents are considered to play key roles in dendritic spiking as well as neurotransmitter release. Sensory neurons of the dorsal root ganglia (DRG) are functionally diverse and contain various neurotransmitters as well as receptors and ion channels. Our previous work demonstrated that both the excitability and transmitter release of sensory neurons are enhanced in sensory neurons isolated from *Nf1*+/– mice compared to the wildtype (Wang et al., 2005; Hingtgen et al., 2006). Although multiple classes of Ca^{2+} channels are expressed in DRG sensory neurons, the contribution of each specific channel subtype to the total Ca^{2+} current in *Nf1*+/– sensory neurons has not been established. To determine this, we used whole-cell patch-clamp recordings and real-time quantitative polymerase chain reaction (qPCR) to assess the extent of different subtypes of calcium channels from small-diameter wildtype and *Nf1*+/– sensory neurons. In this report, we demonstrate that the average peak calcium current (I_{Ca}) densities were not different between the two genotypes. However, N-type currents were significantly larger in *Nf1*+/– neurons although the mRNA levels were not different between the genotypes. These results demonstrate that sensory neurons from *Nf1*+/– mice, exhibit increased N-type Ca^{2+} currents and this likely accounts for the increased release of neuropeptides that occurs in *Nf1*+/– sensory neurons. Part of this work has been published in abstract form (Duan et al., 2010).

EXPERIMENTAL PROCEDURES

Animals

Mice, a C57BL/6J background, were heterozygous for the *Nf1* mutation; these mice were originally created by Dr. Tyler Jacks (Jacks et al., 1994). Mice were housed and bred in the Indiana University Laboratory Animal Research Center and had free access to food and water. These mice were used according to the guidelines in the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Isolation and maintenance of mouse sensory neurons

With some modifications to the protocol developed by Lindsay (1988), sensory neurons were isolated from young adult mice (1–2 months of age). In our studies, both wildtype and *Nf1*+/– mice were littermates. Male mice were killed by putting them in a chamber containing CO_2. The DRG were harvested from the isolated spinal column, the ganglia were placed in a culture dish containing sterilized Pucks solution that was composed of (in mM): 171 NaCl, 6.7 KCl, 1.6 Na_2PO_4, 0.5 KH_2PO_4, 6 D-glucose, and 0.01% phenol red, pH 7.3. The ganglia were placed into a conical tube containing Pucks solution and papain (10 ng/ml); ganglia were digested for 10–12 min at 37 °C after which they were moved to a conical tube containing F-12 medium with 1 mg/ml collagenase 1A and 2.5 mg/ml dispase. After incubation for 10–15 min at 37 °C, the tube was centrifuged at low speed (2000*g*) for 30 s, whereupon the enzyme-containing supernatant was removed. F-12 medium was used to resuspend the pellet, which was then mechanically dissociated with fire-polished pipettes. Cells were plated onto poly-D-lysine- and laminin-coated plastic cover slips. The cells were bathed in F-12 medium supplemented with 10% horse serum, 100 μg/ml normocin, 50 μM 5-fluoro-2′-deoxyuridine, 50 μg/ml penicillin and streptomycin, 2 mM glutamine, 150 μM uridine at 37 °C and 3% CO_2. The cells were used within 12–48 h for electrophysiological recordings. The Animal Use and Care Committee of the Indiana University School of Medicine approved all procedures.

Electrophysiology

The whole-cell patch-clamp recording technique was used as previously described (Wang et al., 2010a). Neurons were bathed in normal Ringers of the following composition (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH adjusted to 7.4 with NaOH. A VC-8 bath perfusion system (Warner Instruments, Hamden, CT, USA) was used to superfuse the recording chamber. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) was used to record whole-cell currents, which were established in normal Ringers. The data were obtained as well as analyzed with the pCLAMP 9.2 suite (Molecular Devices).

To isolate I_{Ca}, neurons were superfused with a Ringers solution composed of (in mM): NMG-Cl 110, tetraethyl ammonium (TEA)-Cl 30, $CaCl_2$ 2, HEPES 10, glucose 10, pH 7.4, adjusted with TEA-OH; 500 nM tetrodotoxin (TTX) was added to this solution on the day of recording. Pipettes used in these recordings were pulled from capillary glass tubing (Model G85165T-4, Warner Instruments); resistances of 1–3 MΩ were determined when filled with the following solution (mM): CsCl 100, EGTA 10, $MgCl_2$ 1, Na_3GTP 0.3, Na_2ATP 4, HEPES 30; pH 7.2, adjusted with CsOH. The data were acquired at 10 kHz and filtered at 5 kHz. Leakage currents were subtracted by using the P/4 protocol. Series resistance was compensated between 60% and 80%. Cell capacitance was determined by using the membrane test feature of Clampex. The peak current amplitude established the current–voltage relation for I_{Ca}. Activation of I_{Ca} was measured by using a holding voltage of −90 mV with voltage steps 200 ms in duration applied at 5-s intervals in +10-mV increments from −90