Upregulation of the T-Type Calcium Current in Small Rat Sensory Neurons After Chronic Constrictive Injury of the Sciatic Nerve

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Submitted 17 September 2007; accepted in final form 16 April 2008

Jagodic MM, Pathirathna S, Jokovic PM, Lee WY, Nelson MT, Naik AK, Su P, Jevtovic-Todorovic V, Todorovic SM. Upregulation of the T-type calcium current in small rat sensory neurons after chronic constrictive injury of the sciatic nerve. J Neurophysiol 99: 3151–3156, 2008. First published April 16, 2008; doi:10.1152/jn.01031.2007. Recent data indicate that peripheral T-type Ca2+ channels are instrumental in supporting acute pain transmission. However, the function of these channels in chronic pain processing is less clear. To address this issue, we studied the expression of T-type Ca2+ currents in small nociceptive dorsal root ganglion (DRG) cells from L4,5 spinal ganglia of adult rats with neuropathic pain due to chronic constrictive injury (CCI) of the sciatic nerve. In control rats, whole cell recordings revealed that T-type currents, measured in 10 mM Ba2+ as a charge carrier, were present in moderate density (20 ± 2 pA/pF). In rats with CCI, T-type current density (30 ± 3 pA/pF) was significantly increased, but voltage- and time-dependent activation and inactivation kinetics were not significantly different from those in controls. CCI-induced neuropathic pain did not significantly change the pharmacological sensitivity of T-type current in these cells to nickel. Collectively, our results indicate that CCI-induced neuropathy did not significantly change the pharmacological sensitivity of T-type current in small DRG neurons. Our finding that T-type currents are upregulated in a CCI model of peripheral neuropathy and earlier pharmacological and molecular studies suggest that T-type channels may be potentially useful therapeutic targets for the treatment of neuropathic pain associated with partial mechanical injury to the sciatic nerve.

INTRODUCTION

For more than two decades, it has been recognized that T-type (low-voltage-activated) Ca2+ channels (T-channels) have a key function in neuronal subthreshold membrane oscillations and spike firing in both the peripheral and CNS (reviewed in Perez-Reyes 2003). However, the function of these channels in sensory and pain transmission (nociception) has only been discovered recently (Todorovic et al. 2001). New data have established the function of T-channels in supporting acute peripheral nociception. Furthermore, pharmacological and molecular downregulation of the function these channels in DRG neurons also supports the notion that T-channels contribute to the chronic pain associated with peripheral axonal injury (reviewed in Jevtovic-Todorovic and Todorovic 2006). However, in spite of the fact that T-type currents are expressed in several subpopulations of nociceptive DRG cells (Coste et al. 2007; Nelson et al. 2005; Todorovic et al. 2001), the cellular basis for the role of T-channels in chronic pain states is poorly understood. To address this issue, we used patch-clamp recordings to study the properties of T-type currents in acutely isolated and intact small nociceptive DRG neurons in animal model of painful peripheral neuropathy induced by chronic constrictive injury of the sciatic nerve.

METHODS

Ethical approval was obtained for all experimental protocols from the University of Virginia Animal Care and Use Committee, Charlottesville, VA. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the U.S. National Institute of Health. Every effort was made to minimize animal suffering and the number of animals used.

Abnormalities in pain perception that are similar to those in humans, such as mechanical and thermal hyperalgesia, as well as mechanical allodynia, have been reported to occur in experimental rat models of mechanical injury of peripheral nerves as a consequence of loose ligation of the sciatic nerve, a CCI (Bennett and Xie 1988). We have published experimental procedures for CCI of the right sciatic nerve (Pathirathna et al. 2005; Todorovic et al. 2004) to induce mechanical injury to peripheral sensory nerve. For the present experiments, we used adult female retired-breeder Sprague-Dawley rats (250–350 g, 6–10 mo old). Control age-matched rats received either no operation ( naïve animals) or sham operation (sham-operated animals). To determine the neuropathic state in CCI-treated rats, we measured thermal nociception in hind paws using thermal radiant heat testing as previously described (Pathirathna et al. 2005; Todorovic et al. 2001, 2004). For all behavioral data, we used ANOVA to compare the effects of CCI on thermal sensation. Subsequent pairwise comparisons between the pre- and post-CCI paw withdrawal latency (PWL) were done if significant P values resulted from two-way ANOVA. When appropriate, alpha levels were adjusted using the Bonferroni procedure (Pathirathna et al. 2005).

Before tissue harvest, rats were deeply anesthetized with isoflurane and rapidly decapitated. For one experiment, we dissected two dorsal root ganglia (DRGs), L4,5 from ligated (right-side) from one CCI or sham-treated rat. In control (untreated, naive) rats, we used bilateral L4,5 DRGs. We chose L4 and L5 DRGs because they contain the cell bodies of the majority of sensory fibers of the sciatic nerve. We prepared dissociated DRG cells and used them within 6–8 h for whole cell recordings as previously described (Todorovic and Lingle 1998). We focused only on small-size cells with an average soma diameter of 15–27 μm (Scroggs and Fox 1992) because many functional studies...
have confirmed that the vast majority of them are nociceptors (Caterina and Julius 2001; McCleskey and Gold 1999).

Recordings were made according to the procedures we described previously, using standard whole cell techniques with acutely dissociated DRG neurons (Jagodic et al. 2007; Nelson et al. 2005, 2007; Todorovic and Lingle 1998) and intact DRG neurons (Nelson et al. 2005). Series resistance ($R_s$) and capacitance ($C_m$) values were taken directly from readings of the amplifier after electronic subtraction of the capacitive transients. Series resistance was compensated to the maximum extent possible (usually ~60–80%). In most experiments, we used a P/5 protocol for on-line leak subtractions.

Drugs were prepared as 100 mM stock solutions of NiCl$_2$ in H$_2$O. The external solution used to isolate Ca$^{2+}$ currents contained (in mM) 10 BaCl$_2$, 152 TEA-Cl, and 10 HEPES adjusted to pH 7.4 with TEA-OH. To minimize contamination of T-type currents with even minimal high-voltage-activated (HVA) components, we used only fluoride (F$^-$)-based internal solution to facilitate HVA Ca$^{2+}$ current rundown; this solution contained (in mM) 135 tetramethyl-ammonium-hydroxide (TMA-OH), 10 EGTA, 40 HEPES, and 2 MgCl$_2$, adjusted to pH 7.2 with hydrofluoric acid (HF). This allowed studies of well-isolated and well-clamped T-type currents in acutely isolated DRG cells (Todorovic and Lingle 1998). For voltage-clamp recordings with intact ganglia, the external solution contained the following (in mM): 140 NaCl, 4 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH; the pipette solution contained the following (in mM): 130 KCl, 5 MgCl$_2$, 1 EGTA, 40 HEPES, 2 Mg-ATP, and 0.1 Na-GTP, adjusted to pH 7.2 with KOH.

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. Unless otherwise indicated, statistical comparisons were made, where appropriate, using an unpaired Student’s $t$-test, Mann-Whitney sum test, signed-rank test and $\chi^2$ test. All quantitative data are expressed as means of multiple experiments ± SE. The percent reductions in peak current at various Ni$^{2+}$ concentrations were used to generate a concentration-response curve. Mean values were fit to the following Hill function

$$PB_{\text{max}} \left[ IC_{50}\left[\text{Ni}^{2+}\right]\right]^n = \frac{PB_{\text{max}}}{1 + \left( IC_{50}\left[\text{Ni}^{2+}\right]\right)^n}$$

where $PB_{\text{max}}$ is the maximal percent block of peak current, $IC_{50}$ is the concentration that produces 50% inhibition, and $n$ is the apparent Hill coefficient for blockade. The fitted value is reported with 95% linear confidence limits. The voltage dependencies of activation and steady-state inactivation were described with single Boltzmann distributions of the following forms

Activation: $G(V) = G_{\text{max}}/(1 + \exp \left[-(V - V_{50})/k\right])$

Inactivation: $I(V) = I_{\text{max}}/(1 + \exp \left[(V - V_{50})/k\right])$

In these forms, $I_{\text{max}}$ is the maximal amplitude of current and $G_{\text{max}}$ is the maximal conductance. $V_{50}$ is the voltage where half of the current is activated or inactivated, and $k$ represents the voltage dependence (slope) of the distribution. The amplitude of T-type current was measured from the peak, which was subtracted from the current at the end of the depolarizing test potential to avoid contamination with residual HVA currents that were present at more positive membrane potentials (typically ~20 mV and higher).

**RESULTS**

Most of the rats developed a stable hyperesthetic state 5–7 days after being subjected to CCI of right sciatic nerve (Pathiratha et al. 2005). We confirmed the presence of CCI-induced thermal hyperalgesia before sacrifice (typically 7–14 days after CCI) for all animals used in voltage-clamp recordings. This was indicated by the reduction in thermal PWLs to 51.20 ± 0.01% of baseline values in right-side (ipsilateral, ligated) paws ($n = 34, P < 0.001$). In contrast, thermal PWLs in these rats in left-side (contralateral, nonligated) paws were not significantly affected: 99.80 ± 0.01% of baseline recorded before surgery (data not shown). Baseline values for PWLs were $10.47 ± 0.08$ s in the right paws and $10.40 ± 0.08$ s in the left paws. A sham treatment did not significantly affect thermal PWLs ≤15 days after the procedure (data not shown).

We recorded from a total of 167 small-size acutely dissociated DRG cells. The average diameter of cell somas was (in $\mu$m): 24.2 ± 0.3 in the control group, 24.3 ± 0.2 in the CCI group, and 24.6 ± 0.9 in the sham group ($P > 0.05$). These included 77 cells from control naïve rats ($n = 22$ animals), 14 cells from sham-operated rats ($n = 2$ animals) and 76 cells from CCI-subjected rats ($n = 34$ animals).

To compare the expression of T-type voltage-gated Ca$^{2+}$ currents in small DRG cells after the induction of CCI, we held cells at −90 mV, then imposed voltage commands of depolarizing pulses from −60 to 0 mV in 10-mV increments. A representative family of inactivating inward currents in small DRG cell from control and CCI-treated animals is depicted in Fig. 1, A and B, respectively. Note that in both cells, T-type Ca$^{2+}$ currents activate with small membrane depolarization, have a characteristic criss-crossing pattern, and display fast and almost complete inactivation during 250-ms-long test potentials. The average current-voltage curves were constructed from similar experiments, which indicated significant enhancement of T-type Ca$^{2+}$ current amplitudes (measured from peak to the end of the depolarizing pulse) in CCI-treated animals; these currents were most prominent at negative membrane potentials and peaked at about ~20 mV (Fig. 1C). To further determine the magnitude of the T-type current increase in CCI rats and to express it as current density, we normalized peak inward currents evoked at −30 mV to the cell capacitance in neurons from sham-operated, CCI-treated, and control rats.

The histograms in Fig. 1D indicate that T-type current density was enhanced ~1.5-fold in DRG cells from CCI rats as compared with cells from control ($P < 0.05$) and sham-operated rats ($P < 0.01$, Mann-Whitney test). Similarly, we found that the average T-current density in small DRG cells per rat had higher values in the CCI group (31.5 ± 4.2 pA/pF, $n = 22$ rats) than in the control group (20.6 ± 2.1 pA/pF, $n = 16$ rats, $P < 0.05$, Mann-Whitney test; data not shown). The average capacitance in these cells was (in pF): 19.4 ± 0.8 for the control group, 15.8 ± 0.7 for the CCI group ($P < 0.01$, $t$-test), and 19.3 ± 1.5 for the sham-operated group (data not shown). We also recorded T-currents from acutely isolated medium-size cells ($n = 18$) from CCI rats ($n = 5$) and found the following: average T-current density, $64 ± 12$ pA/pF; average cell soma diameter, $33.8 ± 4.4 \mu$m; average cell capacitance, $31.4 ± 2.7$ pF; data not shown). None of these parameters was statistically different from those for medium DRG cells in control rats recorded under identical experimental conditions (Jagodic et al. 2007). Next we used data presented in Fig. 1D to generate linear correlation curves from scatter plots of T-type current density against cell capacitance in control and CCI groups. We found significant correlation only in the CCI group ($P < 0.001$), not in the control one ($P = 0.2$, data not shown). These data suggest that CCI-induced increase in T-type current density occurred predominantly in the sub-population of smallest DRG cells.
We also measured time-dependent activation (10–90% rise time, Fig. 1E) and time-dependent inactivation time constants (τ) (single-exponential fit of decaying portion of the current waveforms, Fig. 1F) from current-voltage curves in these cells over the range of test potentials from –50 to 0 mV. We found a small but significant difference between the controls and CCI-treated groups only for inactivation τ at 0 mV (Fig. 1F).

The proportion of cells expressing T-type current was not significantly different in these two groups: control, 69% and CCI, 70% (χ² test, data not shown).

We also tested voltage-dependent (steady-state) inactivation, finding that CCI caused a very small depolarizing shift in the midpoint (V₃₀) of inactivation in these cells. For example, Fig. 2B shows that the inactivation, V₃₀, was about –69 mV in control cells (n = 13) and –66 mV in DRG cells from CCI-induced neuropathic rats (n = 17; P > 0.05). Likewise, the average V₃₀ for T-type channel activation calculated from current-voltage curves was not significantly different in cells from the control (–42 mV, n = 15) and CCI (–41 mV, n = 24) groups (Fig. 2C). In contrast, we recently reported upregulation and a depolarizing shift in voltage-dependent inactivation of T-type channels in a subpopulation of medium-size DRG cells in rats with streptozotocin-induced diabetic neuropathy (Jagodic et al. 2007). Thus it appears that diabetic and mechanical neuropathy may affect T-type channels differently in different subpopulations of DRG cells.
Next we performed experiments to estimate the contribution of T-type current to cellular excitability in these cells. To provide more physiologically relevant data, we performed these recordings using small cells in intact L4-5 DRGs (Nelson et al. 2005) and measured peak inward currents evoked from $V_h = -90$ mV in 5-mV increments. We reasoned that voltage for half-maximal activation of total voltage-gated inward currents in these cells may be shifted to more depolarized potentials in the presence of 100 μM nickel. Indeed, Fig. 3D shows that nickel significantly increased the half-maximal activation threshold from $-40 \pm 1$ to $-31 \pm 3$ mV ($P < 0.01; n = 11$ cells from 4 rats) in CCI but not in DRG cells from sham-operated rats (baseline: $-37 \pm 3$ mV, nickel $-36 \pm 3$ mV; $P > 0.05; n = 8$ cells from 3 rats). Interestingly there was no significant difference in baseline half-threshold activation in the two groups. This could be explained by the concomitant decrease in TTX-resistant Na$^+$ current density reported previously in small DRG cells in rats with CCI (Dib-Hajj et al. 1999). TTX-resistant Na$^+$ channels activate over a range of potentials similar to that which activates T-type channels and are often co-expressed in the same DRG cells (Coste et al. 2007).
DISCUSSION

Here we demonstrate that CCI of the sciatic nerve induces upregulation of Ca$_{v}$3.2 T-type Ca$^{2+}$ currents in small DRG neurons, most of which likely represent the cell somas of classically described peripheral nociceptors. In vitro these cells express functional properties of nociceptors such as responses to capsaicin-, heat-, proton-, and ATP-gated currents (Todorovic et al. 2001) as well as high-threshold mechano-sensory currents (Coste et al. 2007). Thus it is very likely that these T-channel-containing small DRG cells are polymodal nociceptors capable of responding in vivo to noxious heat, chemical, and mechanical stimuli. Recent data indicate that T-type channels have an important function in enhancing the cellular excitability of at least some small DRG cells by reducing the threshold for action potential firing (Nelson et al. 2005) and contributing to Ca$^{2+}$ entry during action potentials (Blaire and Bean 2002; Nelson et al. 2005). In spite of their modest expression in most small DRG cells, increased T-type current density similar to that described here in our CCI model could be sufficient to increase the probability of the firing of action potentials because these cells have high-input resistance, as we have directly demonstrated in our current-clamp recordings using T-type channel modulators and Ca$_{v}$3.2 knock-out mice (Nelson et al. 2007).

It is important to note that remodeling of other voltage- and ligand-gated ion channels that can alter the excitability of the sensory neurons has been proposed to have a critical function in the development and maintenance of neuropathic pain symptoms such as hyperalgesia and allodynia (Campbell and Meyer 2006; Woolf 2004). Thus it is unlikely that changes in T-type current expression in the CCI model are the only culprit but may contribute to complex plasticity and to overall alteration in the cellular excitability of injured sensory neurons. In a simple model of measure of cellular excitability, we found that nickel at concentrations thought to be selective for T-type current significantly increased the threshold for half-maximal activation of total inward current largely carried by voltage-gated Na$^{+}$ channels in these cells from CCI-subjected but not sham-operated rats. These data suggest that upregulated T-type currents may have a more prominent part in lowering threshold for spike firing in small DRG cells from CCI rats than in healthy rats.

Unlike pain that is caused by acute tissue injury (nociceptive pain), neuropathic pain resulting from constrictive nerve damage is a debilitating disorder that is inconsistently responsive to currently available conventional treatments. Of particular interest is our finding that several pharmacological blockers and modulators of T-type channels in vivo alleviate neuropathic pain in CCI. We determined that a series of 5α-reduced neuroactive steroids [e.g., (+)-ECN] are potent and selective blockers of DRG T-type channels in vitro (Todorovic et al. 1998). Furthermore, consistent with our present findings, (+)-ECN had a more potent analgesic effect when injected locally in peripheral receptive fields of sensory neurons in rats with CCI than it did when injected in control rats (Pathiratna et al. 2005). Dogrul and colleagues (2003) found that the preferential T-type channel blockers mibebradil and ethosuximide effectively reverse hyperalgesia and allodynia from CCI. We also found that oxidizing agents that block DRG T-type channel current in vitro are capable of reversing CCI-induced thermal hyperalgesia in vivo (Todorovic et al. 2004). Moreover application of the T-type channel blocker Ni$^{2+}$ blocks ectopic discharges from peripheral nerves in a model of segmental spinal mechanical injury (Liu et al. 2001). Toward this end, specific molecular silencing of Ca$_{v}$3.2 T-type channels in DRG cells with antisense reverses both hyperalgesia and allodynia in rats with CCI (Bourinet et al. 2005).

Surprisingly, in vivo study using Ca$_{v}$3.2 knock-out mice did not find a difference in pain perception in a CCI model (Choi et al. 2006). The exact reason for this discrepancy is not known, but it is possible that developmental elimination of Ca$_{v}$3.2 channels allows compensatory changes that are not feasible during acute downregulation of channel function using pharmacological agents or antisense applications. Interestingly, previous patch-clamp recordings of DRG Ca$^{2+}$ channels in CCI also gave contrasting results. Hogan et al. (2000) reported no change in total inward Ca$^{2+}$ currents in small DRG cells and loss of T-type current in medium-size DRG cells in rats with CCI (McCallum et al. 2003). These authors also reported that in control animals they did not observe T-type currents in any small DRG cells (<29 μm soma diameter). This is in sharp contrast to the results of this and previous studies (Blaire and Bean 2002; Cardenas et al. 1995; Coste et al. 2007; Scroggs and Fox 1992; Todorovic and Lingle 1998; Todorovic et al. 2001), which found that T-type currents are expressed in the majority of small DRG nociceptors and that T-type current in medium-size DRG cells is not affected with peripheral nerve injury (Baccei and Kocsis 2000). Thus it is possible that different experimental conditions or the selection of different cells can account for the different findings with regard to the effect of CCI on expression of Ca$^{2+}$ channels in small and medium DRG cells.

Our present study further implicates T-type channels as possible targets for the treatment of neuropathic pain resulting from mechanical injury to peripheral axons of sensory neurons. Thus blocking T-type channels may offer new therapeutic options for alleviating patients’ suffering from chronic intractable pain resulting from partial mechanical injury of peripheral nerves. Future studies must be focused on the mechanisms of T-type channel alterations in these cells by CCI and in other animal models of painful neuropathy.

GRANTS

This work was supported in part by National Institute of General Medical Sciences Grant ROI-075229 and funds from Department of Anesthesiology at UVA to S. M. Todorovic and funds from the Dr. Harold Carron endowment to V. Jevtovic-Todorovic.

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