Tetrodotoxin-resistant voltage-dependent sodium channels in identified muscle afferent neurons

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Tetrodotoxin-resistant voltage-dependent sodium channels in identified muscle afferent neurons. J Neurophysiol 108: 2230–2241, 2012. First published August 1, 2012; doi:10.1152/jn.00219.2012.—Muscle afferents are critical regulators of motor function (Group I and II) and cardiovascular responses to exercise (Group III and IV). However, little is known regarding the expressed voltage-dependent ion channels. We identified muscle afferent neurons in dorsal root ganglia (DRGs), using retrograde labeling to examine voltage-dependent sodium (NaV) channels. In patch-clamp recordings, we found that the dominant NaV current in the majority of identified neurons was insensitive to tetrodotoxin (TTX-R), with NaV current in only a few (14%) neurons showing substantial (>50%) TTX sensitivity (TTX-S). The TTX-R current was sensitive to a NaV1.8 channel blocker, A803467. Immunocytochemistry demonstrated labeling of muscle afferent neurons by a NaV1.8 antibody, which further supported expression of these channels. A portion of the TTX-R NaV current appeared to be noninactivating during our 25-ms voltage steps, which suggested activity of NaV1.9 channels. The majority of the noninactivating current was insensitive to A803467 but sensitive to extracellular sodium. Immunocytochemistry showed labeling of muscle afferent neurons by a NaV1.9 channel antibody, which supports expression of these channels. Further examination of the muscle afferent neurons showed that functional TTX-S channels were expressed, but were largely inactivated at physiological membrane potentials. Immunocytochemistry showed expression of the TTX-S channels NaV1.6 and NaV1.7 but not NaV1.1. NaV1.8 and NaV1.9 appear to be the dominant functional sodium channels in small- to medium-diameter muscle afferent neurons. The expression of these channels is consistent with the identification of these neurons as Group III and IV, which mediate the exercise pressor reflex.

NaV1.8; NaV1.9; A803467; dorsal root ganglion neurons; exercise pressor reflex

MUSCLE AFFERENTS are important regulators of motor and cardiovascular physiology. The Group I (Aβ) and II (Aδ) afferents provide sensory feedback to control ongoing motor activity (Houk 1974). The Group III (Aδ) and IV (C) afferents mediate the exercise pressor reflex that is normally triggered by our 25-ms voltage steps, which suggested activity of NaV1.9 channels. The majority of the noninactivating current was insensitive to A803467 but sensitive to extracellular sodium. Immunocytochemistry showed labeling of muscle afferent neurons by a NaV1.9 channel antibody, which supports expression of these channels. Further examination of the muscle afferent neurons showed that functional TTX-S channels were expressed, but were largely inactivated at physiological membrane potentials. Immunocytochemistry showed expression of the TTX-S channels NaV1.6 and NaV1.7 but not NaV1.1. NaV1.8 and NaV1.9 appear to be the dominant functional sodium channels in small- to medium-diameter muscle afferent neurons. The expression of these channels is consistent with the identification of these neurons as Group III and IV, which mediate the exercise pressor reflex.

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vascular system (Baccelli et al. 1999; Bakke et al. 2007; Smith et al. 2006). Thus an understanding of the receptors and ion channels that mediate excitability of these muscle afferents could facilitate development of selective treatments to suppress the exercise pressor reflex and normalize cardiovascular function in patients with peripheral vascular disease.

Voltage-dependent sodium (NaV) channels are the source of electrogenesis in excitable cells and contribute to excitability of muscle afferents. There are nine genes that code for NaV channels (Catterall et al. 2005; Rush et al. 2007). Of these, five are known to be expressed in adult peripheral sensory neurons, NaV1.1, 1.6, 1.7, 1.8, and 1.9 (Benn et al. 2001; Rush et al. 2007). These channels can be broadly classified on the basis of their sensitivity to tetrodotoxin (TTX) as TTX resistant (TTX-R) and TTX sensitive (TTX-S). Of the five NaV channels that are present in adult sensory neurons, three are TTX-S, while NaV1.8 and NaV1.9 are TTX-R. NaV1.1 has a widespread distribution in dorsal root ganglion (DRG) neurons, but its function in sensory neurons has not been fully elucidated (Rush et al. 2007). However, NaV1.1 is localized to the initial axon segment and nodes of Ranvier in spinal neurons (Duflocq et al. 2008). NaV1.6 and 1.7 are necessary for action potential initiation and transmission in both central and peripheral neurons (Rush et al. 2007). NaV1.8 is present in small to medium unmyelinated and thinly myelinated DRG neurons (Djouhri et al. 2003; Renganathan et al. 2001) and is well studied for its role in nociception and chronic pain (Dong et al. 2007; Jarvis et al. 2007; Zimmermann et al. 2007). NaV1.9 is primarily expressed in unmyelinated DRG neurons and has been shown to play a role in nociception (Fang et al. 2002).

Little is known regarding the NaV channels involved in electrogenesis of muscle afferents. Here we investigated the NaV channels expressed in muscle afferent neurons that were identified by retrograde transport of a dye injected into the triceps surae muscle. TTX-R channels appear to be the dominant NaV channel type in the majority of muscle afferent somas from which we recorded. TTX-S channels are also present but appear to be largely inactivated at physiological resting membrane potentials. Our evidence supports the expression of both NaV1.8 and 1.9 in these neurons. NaV1.8 is highly expressed in small and medium C and Aδ sensory neurons (Djouhri et al. 2003; Puhl and Ikeda 2008), which suggests that these muscle afferents were Group III (Aδ) and IV (C) that mediate the exercise pressor reflex (Kaufman and Hayes 2002; Kaufman et al. 1983).

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MATERIALS AND METHODS

Labeling of muscle afferent neurons. Adult male Sprague-Dawley rats were obtained from Hill Top Laboratories (Scottsdale, PA). The rats were housed in a temperature-controlled room (24 ± 1°C) with a 12:12-h light dark cycle and fed a standard diet and tap water ad libitum. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Retrograde labeling with the lipophilic dye 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) was used to identify muscle afferent neurons. Rats (wt 150 – 400 g) were anesthetized with isoflurane in an 1:1 mixture of O2 and N2 and a vaporizer (Harper and Lawson 1985) with a specific membrane capacitance of 1 μF/cm² (Ho and O’Leary 2011). Dose-response data were fit by using Hill’s equation to obtain the IC50. Group data are calculated as means ± SD throughout this report. Student’s t-test was calculated to determine significant differences (P < 0.05).

Immunochemistry. Neurons were fixed with 4% formaldehyde and permeabilized with 2% Tween 20 in phosphate-buffered saline (PBS); normal goat serum was used for blocking. Neurons were incubated overnight with primary antibody for NaV1.3 (mouse, Abcam, Cambridge, MA) or NaV1.1, NaV1.6, NaV1.7, or NaV1.9 (rabbit, Alomone Labs, Jerusalem, Israel) (1:500). Control neurons were labeled with secondary antibody, which was Alexa Fluor 488 IgG goat anti-rabbit (Alomone Labs, Jerusalem, Israel) (1:500). Control neurons were imaged after a 1-h wash in PBS with 0.1% Tween 20. DiI-labeled DRG neurons (see Fig. 4) were identified with a Nikon Diaphot microscope with epifluorescence and were voltage-clamped with the whole cell configuration to obtain the IC50.

RESULTS

TTX sensitivity in muscle afferent neurons. DiI-labeled muscle afferent neurons were selected for recording (see example in Fig. 4). As a first step toward identifying the NaV currents expressed in these neurons, we measured the effect of 300 nM TTX (Fig. 1C). This concentration will block all TTX-S channels without affecting TTX-R channels (Blair and Bean 2002, 2003; Catterall et al. 2005; Elliott and Elliott 1993; Ho and O’Leary 2011; Ogata and Tatebayashi 1993; Renganathan et al. 2001; Roy and Narahashi 1992). Using HP of −80 mV, we found only a few muscle afferent neurons (6/43) that showed strong block (>50%) by TTX. These neurons had cell diameters ranging from 35 to 47 μm, and we have termed these cells TTX-S. The NaV currents from these cells tended to activate and inactivate rapidly (Fig. 1A), with an average rise time (10–90% of maximum current) of 0.25 ± 0.22 ms and a fast inactivation time constant (τ) of 1.1 ± 1.3 ms (mean ± SD, n = 6). The other neurons (37/43) showed a much smaller NaV current block by TTX (<30%) at a HP of −80 mV, and we termed these cells TTX-R (Fig. 1C). The rise time of the NaV current in TTX-R neurons (Fig. 1B) was significantly larger than that of TTX-S neurons, with the 10–90% rise time = 0.63 ± 0.13 ms (P < 0.05 relative to TTX-S). The speed of
inactivation was also significantly slower compared with TTX-S current, with a fast inactivation $\tau = 3.2 \pm 1.6$ ms (mean $\pm$ SD, $n = 37$, $P < 0.05$) at 10 mV.

The diameter of the TTX-R muscle afferent neurons had a range of 27–50 $\mu$m, which spans that of the TTX-S neurons (35–47 $\mu$m; Fig. 1C). The majority of neurons (54%) had a diameter between 30 and 40 $\mu$m, while 19% had diameters in the 20–30 $\mu$m range and 27% had diameters in the 40–50 $\mu$m range. This distribution matches that found by Hu and McLachlan (2003), where the majority of muscle afferent neurons had diameters in the 30–40 $\mu$m range ($\sim$38%), with fewer in the 20–30 ($\sim$22%) and 40–50 ($\sim$23%) $\mu$m ranges.

For comparison, we examined cutaneous afferent neurons (see MATERIALS AND METHODS) to determine the $\mathrm{Na}_v$ current TTX sensitivity (Fig. 1D). The 13 neurons recorded (HP $-80$ mV) only 1 showed strong TTX sensitivity, while TTX block of $\mathrm{Na}_v$ current was $<20\%$ in the other 12 cutaneous afferent neurons (Fig. 1D). The diameter of TTX-R cutaneous afferent neurons ranged from 22 to 48 $\mu$m, with the majority of neurons having diameters $<35$ $\mu$m. The diameter of the single TTX-S neuron was 22 $\mu$m. Figure 1D shows that the neuronal diameters of the cutaneous afferents tended to be smaller than those of the muscle afferents (Fig. 1C) from which we recorded. The smaller diameter of cutaneous versus muscle afferent neurons is consistent with previous findings (Hu and McLachlan 2003).

**TTX-S muscle afferent neurons.** The $\mathrm{Na}_v$ current in TTX-S muscle afferent neurons ($n = 6$) was further examined by comparing TTX block at different times during the 25-ms step. The peak current was blocked by 96 $\pm$ 1.5% in response to 300 nM TTX, while the current at the end of the 25-ms step was not significantly affected ($1.2 \pm 1.4\%$) (Fig. 2A and C). The complete block of the inactivating $\mathrm{Na}_v$ current in these neurons demonstrates that 300 nM TTX blocks all TTX-S channels. The time course data show that TTX rapidly blocked peak current with recovery over a washout period of 10 min (Fig. 2B). We would have liked to investigate the source for the small TTX-R sustained current in these cells, but the low frequency of encountering such neurons prevented a more detailed study. However, the absence of inactivation over the 25-ms step suggests that $\mathrm{Na}_v1.8$ is not the source for this TTX-R current.

A803467 blocked current in TTX-R muscle afferent neurons. TTX-R $\mathrm{Na}_v1.8$ channels are expressed in DRG neurons (Bulaj et al. 2006; Jarvis et al. 2007), and we hypothesized that a large fraction of the $\mathrm{Na}_v$ current in our TTX-R muscle afferent neurons was generated by $\mathrm{Na}_v1.8$ channel activity. This hypothesis was supported by the slower activation and inactivation kinetics of the $\mathrm{Na}_v$ current in TTX-R versus TTX-S neurons (Abdulla and Smith 2002; Blair and Bean 2002). We further tested this hypothesis by examining the effect of the $\mathrm{Na}_v1.8$ blocker A803 (Jarvis et al. 2007). A concentration of 300 nM A803 more effectively blocked peak $\mathrm{Na}_v$ current ($41 \pm 15\%$) while inducing a smaller block of current at the end of the 25-ms step ($22 \pm 9\%$) in neurons ($n = 22$) exposed for at least 6 min (Fig. 3, A and C). The block of peak current by 300 nM TTX was only $6 \pm 7\%$ in the same 22 neurons, which shows that these muscle afferent neurons belonged to the TTX-R group (Fig. 3C). For the TTX-R cutaneous afferent neurons, $\mathrm{Na}_v$ current block was $2 \pm 4\%$ by 300 nM TTX and $65 \pm 12\%$ by 300 nM A803 ($n = 12$). This A803 block is larger than that reported above for muscle afferent neurons but overlaps the A803 block of muscle afferent $\mathrm{Na}_v$ current obtained in a separate set of experiments (see below).

The time course for 300 nM A803 block of $\mathrm{Na}_v$ current in muscle afferent neurons was slow (Fig. 3B), with the average blocking $\tau = 4 \pm 3$ min (mean $\pm$ SD, $n = 22$). Unfortunately, we were not able to obtain recovery of $\mathrm{Na}_v$ current during washout of A803 (Fig. 3B). The average recovery was only $13 \pm 9\%$ ($n = 16$) at 6 min after return to control external solution. Thus A803 was poorly reversible in our recordings.
The absence of recovery of Na\textsubscript{v} current from A803 forced us to use a cumulative method to assess the A803 dose-response relationship, in which concentrations were added in increasing order. The slow time course of block also complicated this measurement since we wanted to obtain data from multiple doses in the same neuron. We used an isochronic measurement technique in which the effect of each concentration was measured at the end of a 6-min application. These two methods have been previously combined to successfully assess the dose-response relationship of a slow ion channel blocker (Boland et al. 1994). TTX-R muscle afferents were exposed to 30, 100, 300, and 1,000 nM A803. Fractional block was plotted against A803 concentration and fitted with the Hill equation (Fig. 3D), which yielded an IC\textsubscript{50} of 186 nM, a Hill coefficient of 0.7, and a 65% maximum block (Fig. 3D).

Our IC\textsubscript{50} of 186 nM is close to the previously published IC\textsubscript{50} (140 nM) determined from rat DRG neurons (Jarvis et al. 2007), which supports the hypothesis that A803 is blocking Na\textsubscript{v}1.8 channels in our experiments. The presence of Na\textsubscript{v}1.8 channels in the muscle afferents was further investigated by using immunocytochemistry to gauge the expression of these channels (Fig. 4). Consistent with our recordings, the majority of identified muscle afferents (21/28, 75%) were stained with the Na\textsubscript{v}1.8 antibody. The distribution of cell diameters is shown for muscle afferent neurons that were positively (Fig. 4, top left) and negatively (Fig. 4, bottom left) stained by the Na\textsubscript{v}1.8 antibody. The unstained muscle afferents tended to have smaller diameters than the labeled muscle afferent neurons, with the majority of unstained neurons having diameters <30 \textmu m. The Na\textsubscript{v}1.8-labeled muscle afferent neurons had diameters >20 \textmu m with peak numbers in the 30–40 \textmu m range, which fits with our electrophysiology data. Interestingly, even very large muscle afferent neurons (diameter > 40 \textmu m) were stained with the Na\textsubscript{v}1.8 antibody, which also supports our electrophysiological findings.

Recovery of TTX-S Na\textsubscript{v} channels at hyperpolarized voltages. The absence of TTX-S currents was puzzling since previous studies have demonstrated strong block of muscle afferent transmission by TTX (Tsuchimochi et al. 2011). It has previously been demonstrated in DRG neurons that TTX-S Na\textsubscript{v} current can inactivate at voltages hyperpolarized to TTX-R channels (Leffler et al. 2002), and hyperpolarizing prepulses were shown to enhance TTX-S block.

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have been used to reveal TTX-S currents (Hong et al. 2004). We tested the idea that the TTX-S currents in our muscle afferent neurons were largely inactivated at our HP of −80 mV by hyperpolarizing the HP to −100 mV and retesting with 300 nM TTX. This hyperpolarization recovered a current that tended to activate more rapidly than the TTX-R current and was completely inactivated by the end of the 25-ms voltage step (Fig. 5A). Thus the amplitude of peak current was increased 40 ± 45% by hyperpolarizing the HP from −80 to −100 mV (n = 5), but current at the end of the step was not changed (12 ± 21%). From a HP of −80 mV, 300 nM TTX induced only a small block of peak current (4 ± 2%), but that block was significantly increased to 35 ± 15% (P < 0.05) upon changing the HP to −100 mV in the same five muscle afferent neurons (Fig. 5B). The diameters of these neurons ranged from 27 to 40 μm (mean 32 μm). TTX-S NaV channels are functionally expressed along with TTX-R channels in muscle afferent neurons but are largely inactivated at a HP of −80 mV.

We were interested to determine which TTX-S NaV channels were expressed in muscle afferent neurons. Previous work has demonstrated that NaV1.1, 1.6, and 1.7 are the major TTX-S channels expressed in postnatal sensory neurons (Ho and O’Leary 2011; Lai et al. 2003; Rush et al. 2007). We found that muscle afferent neurons from adult rats were stained with antibodies for NaV1.6 and NaV1.7 (Fig. 6). For the DI-positive muscle afferent neurons, 8 of 10 were positive for NaV1.6 staining and 5 of 7 were positive for NaV1.7 staining (separate experiments). The eight NaV1.6-positive neurons had diameters ranging from 23 to 35 μm, while the two NaV1.6-negative neurons had diameters of 34 and 56 μm. For NaV1.7, the diameters of the five positive neurons ranged between 26 and 36 μm, while the two negative neurons were 33 and 34 μm. In contrast to NaV1.6 and NaV1.7, none of the muscle afferent neurons (29–37 μm; 0/6) was positively stained by the NaV1.1 antibody (Fig. 7). Thus NaV1.6 and NaV1.7 appear to be the dominant TTX-S channels expressed in small to medium-sized muscle afferent neurons.

A component of TTX-R current is A803 resistant. The peak TTX-R current was blocked on average by ~40% by 300 nM A803, while the current at the end of the voltage step was reduced by only ~20% (Fig. 3C). A803 is relatively specific for NaV1.8 channels (Jarvis et al. 2007), which suggests that a small number of NaV1.8 channels remain active at the end of the 25-ms step. Since this end current is completely TTX insensitive, we hypothesized that at least part of the A803-insensitive current was generated by NaV1.9 channel activity. Consistent with this idea, the NaV1.9 currents are slowly inactivating (Coste et al. 2007) and the A803 resistant current in muscle afferent neurons is largely sustained over the 25-ms voltage step (Fig. 3A). As a first test of this idea, we examined the Na+ sensitivity of TTX-R current by switching the external Na+ concentration from 50 to 0 mM (in the presence of 300 nM TTX), which blocked peak current by nearly 100% but blocked end current by only ~70% (Fig. 8, A and B). Thus NaV channels appear to generate only ~70% of the sustained current. In separate set of experiments, we tested the sodium sensitivity of the current in the presence of 300 nM TTX and 300 nM A803. TTX blocked peak NaV current by 5 ± 4%, while A803 blocked current by 65 ± 5% in this set of experiments (n = 4). In the zero-Na+ external solution, the TTX-A803-resistant current was blocked by 87 ± 8% at peak concentration.
and by 37 ± 11% (n = 4) at the end of the step (Fig. 8C). The apparent incomplete block of NaV1.8 channels by A803 (Fig. 3, C and D) complicates the determination of which NaV channel is the source for this Na⁺-sensitive current. However, it is likely that part of the sustained TTX-A803-resistant current is generated by NaV1.9 channel activity. Further support was obtained by staining muscle afferent neurons with a NaV1.9 antibody. We found positive NaV1.9 staining in all 10 muscle afferent neurons (diameters 22–45 μm) examined (Fig. 8, D and E).

The Na⁺-insensitive current is largely sensitive to Cd²⁺.

Several properties of the current observed in zero-Na⁺ external solution suggested that the source could be voltage-dependent calcium (CaV) channel activity, including slower current activation than that of NaV current, and the current was noninactivating over the 25-ms step (Fig. 9A). It seems likely that a small current could arise from Mn²⁺ permeation, since we have previously noted measurable current through CaV2.2 channels carried by 5 mM Mg²⁺ or 5 mM La³⁺ (V. Yarotskyy and K. S. Elmslie, unpublished observations). Since there are multiple CaV channels expressed in DRG neurons (Lu et al. 2010), we chose the broad-spectrum CaV channel blocker Cd²⁺ to test CaV channel involvement. In zero Na⁺, 300 μM Cd²⁺ blocked 80% of the current measured at the end of the 25-ms step (Fig. 9, A and B). The dose-response relationship was well fit by the Hill equation to yield a Hill coefficient of 1.1 and an IC₅₀ of 16 μM, which is consistent with CaV channel block (Carbone et al. 1990; Lansman et al. 1986). NaV
channels are blocked by Cd\(^{2+}\), but the IC\(_{50}\) is typically >100 μM (Coste et al. 2007; Ikeda et al. 1986; Sheets and Hanck 1992). When examined in 50 mM Na\(^{+}\) external solution, 300 μM Cd\(^{2+}\) blocked only a small fraction (35%) of the end current, with an IC\(_{50}\) of 9 μM and a Hill coefficient of 0.8 (Fig. 9, C and D), which are both similar to those obtained in zero-Na\(^{+}\) external solution. Thus the same contaminating Ca\(_{V}\) current appears to be present in external Na\(^{+}\). One surprise was that an additional component of Cd\(^{2+}\) block was not observed when Na\(_{V}\) currents were recorded (50 mM Na\(^{+}\) vs. 0 Na\(^{+}\) external), since it was reported previously that Na\(_{V}\)1.9 current was blocked by Cd\(^{2+}\) with an IC\(_{50}\) of 233 μM (Coste et al. 2007).
Identification of the TTX-S Na\textsubscript{V} channels expressed in muscle afferent neurons was done by immunocytochemistry. Previous work has demonstrated expression of TTX-S Na\textsubscript{V}1.1, 1.6, and 1.7 channels in adult sensory neurons (Ho and O’Leary 2011; Lai et al. 2003; Rush et al. 2007). Using antibodies directed against these channels, we found strong labeling by the Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 antibodies but no labeling of muscle afferent neurons by the Na\textsubscript{V}1.1 antibody. We would have liked to support this finding by using pharmacological agents to identify functional channels. Unfortunately, the specific blockers are not available to differentiate among TTX-S channels. However, recent work shows that the use of multiple \mu-conotoxins may allow the identification of functional TTX-S channels (Wilson et al. 2011).

Two recent reports demonstrate strong suppression of the exercise pressor reflex by TTX applied either directly to the dorsal root (Tsuchimochi et al. 2011) or injected into the DRGs (Wang et al. 2011), which demonstrates that the TTX-S channels are active at least in the axons of the Group III and IV afferents. While we cannot yet explain the differences between our study and those examining axonal responsiveness, it is possible that axonal TTX-S Na\textsubscript{V} channels could be associated with different auxiliary subunits that could depolarize the inactivation vs. voltage relationship relative to those channels.

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expressed in the soma. Previous work has demonstrated that coexpression of NaV β-subunits can shift the NaV channel inactivation-voltage relationship (Johnson and Bennett 2006; Johnson et al. 2004; Vijayaraghavan et al. 2004). For example, coexpression of the TTX-S channel NaV1.7 with the NaV β1-subunit shifts voltage-dependent inactivation ~20 mV depolarized to increase channel availability (Klugbauer et al. 1995).

Noninactivating TTX-R NaV current. A portion of the sodium current did not inactivate during our 25-ms voltage steps and was insensitive to TTX. This current was also largely insensitive to A803. This combination of traits was consistent with NaV1.9 as the source for the noninactivating current (Coste et al. 2007; Cummins et al. 1999, 2000). Unfortunately, NaV1.9 channel-specific blockers do not exist, which prevented the pharmacological assessment of these channels. As an alternative, we assessed the Na\(^+\) sensitivity of this current and found a 70% reduction of end current in our zero-Na\(^+\) external solution, as expected for NaV1.9. The remaining sustained current was Na\(^+\) insenstive and likely resulted from the activity of CaV channels since this current was strongly blocked by Cd\(^{2+}\). We also used immunocytochemistry to assess expression of NaV1.9 in muscle afferent neurons and found that all of the neurons examined were immunoreactive for NaV1.9. Together these results support the functional expression of NaV1.9 in NaV1.8-expressing muscle afferent neurons.

Previous work has demonstrated NaV1.9 expression in Aδ (Group III) and C (Group IV) afferent neurons (Fang et al. 2002). The voltage-dependent and kinetic properties of NaV1.9 channels suggest that their activity contributes to resting membrane potential and action potential initiation, but the activity of these channels alone does not generate action potentials (Cummins et al. 2007; Herzog et al. 2001). Thus enhancement of NaV1.9 channel activity can increase neuronal excitability (Baker 2005), and knockdown of these channels reduces inflammation-induced hyperalgesia (Amaya et al. 2006; Loliigner et al. 2011).

NaV1.8 channels generate the main TTX-R current. The majority of identified muscle afferent fibers from which we recorded (27- to 50-μm diameter) expressed a TTX-R current that activated and inactivated significantly more slowly than the TTX-S currents. These properties are consistent with the identification of the source for this current as NaV1.8 channels (Blair and Bean 2002), which was further supported by the sensitivity of the current to the NaV1.8 blocker A803 (Jarvis et al. 2007). We also used immunocytochemistry to show that the majority of identified muscle afferent neurons were labeled with a NaV1.8 antibody. One surprise was that the muscle afferent neurons not labeled by the NaV1.8 antibody had diameters less than ~30 μm, with 50% of neurons (3/6) with diameters between 25 and 30 μm not labeled. This contrasts with our electrophysiology data, since all six muscle afferent neurons with diameters in the 25–30 μm range expressed TTX-R current that was blocked by A803. One possible explanation for this discrepancy is that we did not record enough neurons in this size range to observe TTX-S neurons, but this seems unlikely if 50% of the neurons fail to express NaV1.8. Another possibility is that the smaller neurons are not permeabilized by our procedure as well as the larger neurons, which would limit antibody access to the intracellular epitope.

Our procedure must permeabilize the neuron to allow antibody access, but we lose DiI labeling if there is too much permeabilization. More work is required to investigate the NaV channels expressed in these smaller muscle afferent neurons.

Both our electrophysiology and immunocytochemistry experiments found strong expression of NaV1.8 channels in medium (30–40 μm)- and large (>40 μm)-diameter muscle afferent neurons. Previous work has shown preferential expression of NaV1.8 channels in small-diameter (<35 μm) C and Aδ neurons, but these were largely cutaneous afferents (Djouhri et al. 2003) and muscle afferents have been shown to have larger diameters than cutaneous afferents (Hu and McLachlan 2003). Our electrophysiological recordings support the larger diameter for muscle afferent neurons. The majority of the cutaneous afferents from which we recorded had diameters <35 μm, while the majority of muscle afferent neurons had diameters >30 μm. Considering this and the fact that medium-sized muscle afferent neurons (30–40 μm) express both NaV1.8 and NaV1.9, it seems that the size range of Group III (Aδ) and Group IV (C) muscle afferent neurons is larger than that for cutaneous afferents.

The expression of NaV1.8 by large-diameter (>40 μm) muscle afferent fibers was surprising but was a consistent result in both our electrophysiology and immunocytochemistry data. However, there is increasing evidence that NaV1.8 channels can be expressed in a wide range of sensory neurons, including Aβ afferents. In a study of human DRG, postmortem samples (male 57–72 yr old) showed NaV1.8 antibody staining in up to 80% of large-diameter neurons (60–80 μm) and staining in afferents (Aβ fibers) innervating cutaneous Meissner’s corpuscles (Coward et al. 2000). In addition, animal studies have demonstrated expression of NaV1.8 in large-diameter DRG neurons in mouse (48%, diameter >40 μm; Renganathan et al. 2000) and rat (39%, area >1,200 μm²; Novakovic et al. 1998). This was further supported in a mouse study demonstrating NaV1.8 expression in DRG neurons expressing Neurofilament 200 (a marker of large-diameter neurons) and in sensory afferents innervating Meissner’s corpuscles (Aβ fibers) (Shields et al. 2012). Thus at least some Group II (Aβ), and perhaps Group I, muscle afferent neurons appear to express NaV1.8.

A803 blocked on average only ~50% of the peak current at the highest concentration used (1 μM). We were reluctant to use higher concentrations since previous work determined the IC\(_{50}\) to be 140 μM for rat NaV1.8 and showed that the block became nonselctive at higher concentrations (Jarvis et al. 2007). Jarvis et al. (2007) demonstrated that block by A803 was incomplete at inactivating voltages hyperpolarized to ~70 mV, which suggests that the inactivation state of NaV1.8 channels is a critical factor. Thus our ~80 mV HP likely limited the maximal block we could achieve using A803. The impact of channel inactivation could also explain the large variance we found in A803 block of NaV current (e.g., Fig. 3D), since small changes in channel inactivation state could have a large impact on block. It is also likely that A803-insensitive NaV currents such as NaV1.9 and TTX-S currents further limited the maximal block (most A803 block was measured in the absence of TTX). While these factors impacted our ability to determine the full magnitude of NaV1.8 current in these neurons, it is clear that NaV1.8 channels...
generate a substantial fraction (50% or more) of the total NaV current in these identified muscle afferents.

NaV_{1.8} channels play a dominant role in action potential generation in the soma of small-diameter neurons (Blair and Bean 2002; Renganathan et al. 2001; Rush et al. 2007). Expression of NaV_{1.8} is strong in soma of these neurons (Djouhri et al. 2003), but expression is normally low in the axons (Gold et al. 2003; Pinto et al. 2008). This pattern of expression appears to be mimicked by muscle afferents. We demonstrated strong NaV_{1.8} expression in the soma of small to medium sized identified muscle afferents (likely Group III and IV), while TTX-S channels appear to dominate action potential propagation in Group III and IV axons (Tsuchimochi et al. 2011; Wang et al. 2011), since axonal application of TTX blocked the exercise pressor reflex. However, NaV_{1.8}, along with NaV_{1.7} and NaV_{1.9}, channels have been shown to be expressed in most cutaneous free nerve endings (presumably nociceptors), which suggests that these channels play a role in sensory transduction (Persson et al. 2010). Thus, even though NaV_{1.8} channels do not participate in axonal action potential propagation in nondisease states, they could play an important role in action potential initiation.

Role for NaV_{1.8} channels in the exercise pressor reflex. NaV_{1.8} has been shown to play a role in increasing excitability of nociceptors in chronic nerve injury models of neuropathic pain (Gold et al. 2003; Roza et al. 2003; Thakor et al. 2009). In addition, A803 and another NaV_{1.8} blocker, μO-conotoxin MrVIB, produce analgesia in animal models of neuropathic changes in axonal NaV channel distributions. One possible whether longer durations of muscle ischemia could affect

Changes are typically measured 7–10 days after injury (Gold et al. 2006; Ekberg et al. 2006; Jarvis et al. 2007). Further support comes from analgesic effects produced by knockdown of NaV_{1.8} with antisense RNA in animal models of neuropathic pain (Joshi et al. 2006; Ruangsri et al. 2011). Together these results suggest that chronic disease states could alter the distribution of NaV_{1.8} in Group III and IV afferents to increase excitability of the exercise pressor reflex. However, one model of peripheral vascular disease, 72-h muscle ischemia, failed to reduce the effect of TTX to block the exercise pressor reflex (Tsuchimochi et al. 2011). While this model does increase cardiovascular responses to muscle contraction (Tsuchimochi et al. 2010), it is possible that 72 h is insufficient to produce changes in NaV_{1} channel distribution in Group III and IV neurons. Chronic nerve injury models that do produce such changes are typically measured 7–10 days after injury (Gold et al. 2003; Jarvis et al. 2007). It would be interesting to see whether longer durations of muscle ischemia could affect changes in axonal NaV_{1} channel distributions. One possible mechanism is the release from the damaged tissues of inflammatory mediators such as tissue necrosis factor-α (TNF-α) and chemokine CCL2, which have both been shown to increase NaV_{1.8} channel activity in uninjured sensory neurons (Belkouch et al. 2011; He et al. 2010).

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