Na$_v$1.6 channels generate resurgent sodium currents in spinal sensory neurons

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Abstract The Na$_v$1.6 voltage-gated sodium channel has been implicated in the generation of resurgent currents in cerebellar Purkinje neurons. Our data show that resurgent sodium currents are produced by some large diameter dorsal root ganglion (DRG) neurons from wild-type mice, but not from Na$_v$1.6-null mice; small DRG neurons do not produce resurgent currents. Many, but not all, DRG neurons transfected with Na$_v$1.6 produce resurgent currents. These results demonstrate for the first time the intrinsic ability of Na$_v$1.6 to produce a resurgent current, and also show that cell background is critical in permitting the generation of these currents.

1. Introduction

Voltage-gated sodium channels underlie the generation of action potentials in excitable cells. Nine different voltage-gated sodium channels have been identified in mammals [1,2]. Many of these channels have specific developmental, tissue or cellular distributions [3], and expression of recombinant channels indicates that different channels can have distinct functional properties [4–6]. Differences in the properties of voltage-gated sodium channels may be important determinants of the integrative and firing properties of neurons.

Resurgent sodium currents are an unusual type of sodium current that reactivate during mild repolarizations following depolarization to positive potentials and may be critical in determining the firing patterns of cerebellar Purkinje neurons [7]. While classic sodium channel tail currents activate instantaneously and decay rapidly, resurgent currents show much slower kinetics and are thought to arise from a distinct inactivation mechanism [8]. A 90% reduction of resurgent sodium currents in Purkinje neurons from Na$_v$1.6 null mice strongly suggested that Na$_v$1.6 underlies most of this current [9]. Na$_v$1.6 channels are expressed in many different neuronal populations including dorsal root ganglion (DRG) neurons [1,3,10]. Therefore, we asked whether DRG neurons could generate resurgent currents. Our results show that resurgent currents can be recorded from native DRG neurons and show that Na$_v$1.6 channels underlie the generation of these currents in these neurons. Part of this work has been presented in abstract form [11].

2. Methods

2.1. Culture of DRG neurons

DRG neurons were cultured as previously described [12]. Briefly, L4 and L5 DRG ganglia were harvested from wild-type mice, Na$_v$1.8-null mice [13] and Na$_v$1.6-null mice [14]. DRGs were treated with collagenase A (1 mg/ml) for 25 min, and collagenase D (1 mg/ml) and papain (30 U/ml) for 25 min, dissociated in DMEM medium supplemented with 10% fetal bovine serum and plated on glass coverslips.

2.2. Biolistic transfection of mouse DRG neurons

The Helios Gene Gun (Bio-Rad Laboratories) was used to transfect DRG neurons with recombinant sodium channels, as previously described [5,6]. To facilitate isolation of Na$_v$1.6 and Na$_v$1.4 currents, our studies utilized cDNA constructs that encode tetrodotoxin-resistant (TTX-R) versions of Na$_v$1.6 (Na$_v$1.6r), and Na$_v$1.4 (Na$_v$1.4r) [15,16]. For these experiments Na$_v$1.8-null neurons were kept under standard tissue culture conditions for 5–7 days before biolistic transfections. We previously showed that Na$_v$1.8-null DRG neurons do not express fast or slow-inactivating TTX-R sodium currents [5,17]. Some Na$_v$1.8-null DRG neurons express persistent TTX-R sodium currents [17], but because these currents fall below 1 nA after several days in culture and run down quickly in whole-cell recording configuration, they are not significant under the recording conditions used in the present study. Neurons were cotransfected with green fluorescent protein (GFP). Electrophysiologic studies were conducted 18–48 h after transfection; most of the cells that expressed GFP also expressed fast-inactivating recombinant TTX-R sodium currents.

2.3. Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were conducted at room temperature (~21 °C) using an EPC-9 amplifier (HEKA Electronic, Germany) as previously described [4]. Fire-polished electrodes were fabricated from 1.7-mm capillary glass using a Sutter P-97 puller (Novato, CA). Voltage errors were minimized using low resistance pipettes (0.8–1.5 MΩ) and 80–90% series resistance compensation. Linear leak currents were subtracted using –P/5 pulse protocols applied before the test protocol for all voltage clamp recordings. The pipette solution

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contained (in mM): 140 CsF, 1 EGTA, 10 NaCl and 10 HEPES (pH 7.3). The standard bathing solution was (in mM) 140 NaCl, 3 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 0.05 CdCl$_2$, and 10 HEPES (pH 7.3). Osmolarity of all solutions was adjusted to 310 mosM using glucose. Data were typically low-pass filtered at 4 kHz and sampled at 20 kHz. Data were analyzed using the Pulsefit (HEKA, Germany) and Origin (Microcal, Northampton, MA) software programs. Unless otherwise noted, statistical significance was determined by $P < 0.05$ using an unpaired t test. Results are presented as means ± S.E.M.

3. Results

3.1. Resurgent sodium currents in native DRG neurons

To determine whether DRG neurons express TTX-sensitive (TTX-S) resurgent sodium currents, we recorded from wild-type mouse DRG neurons, 24 h after culture, using a strong depolarization (20 ms at +30 mV) followed by intermediate repolarization pulses to voltages ranging from −30 to −70 mV (for 100 ms). Resurgent sodium currents were not observed in small (<35 μm diameter) DRG neurons ($n = 8$; Fig. 1A). However, we recorded large (2.1 ± 0.4% of peak current amplitude) resurgent sodium currents from some larger (35–50 μm diameter) DRG neurons. Twelve out of 27 large diameter DRG neurons exhibited resurgent currents (Fig. 1B); some large diameter neurons did not produce resurgent currents (Fig. 1C). To further investigate the origin of these resurgent currents, we recorded from Na$_v$1.8-null mouse DRG neurons, which do not express the slowly-inactivating TTX-R sodium current generated by Na$_v$1.8. While none of the small diameter (<35 μm) Na$_v$1.8-null DRG neurons exhibited resurgent currents ($n = 8$), 5 out of 11 large diameter Na$_v$1.8-null DRG neurons did (Fig. 2A). The voltage-dependence of the resurgent current recorded from DRG neurons is similar to that recorded from cerebellar Purkinje neurons [7,9]. Four of the cells exhibiting resurgent currents were exposed to 500 nM TTX and in each case TTX blocked the resurgent current (Fig. 2C), as it did in wild-type neurons (data not shown). Resurgent currents were never recorded in the presence of TTX in either large or small diameter neurons. These data indicate that TTX-sensitive channels generate resurgent sodium currents in large diameter DRG neurons.
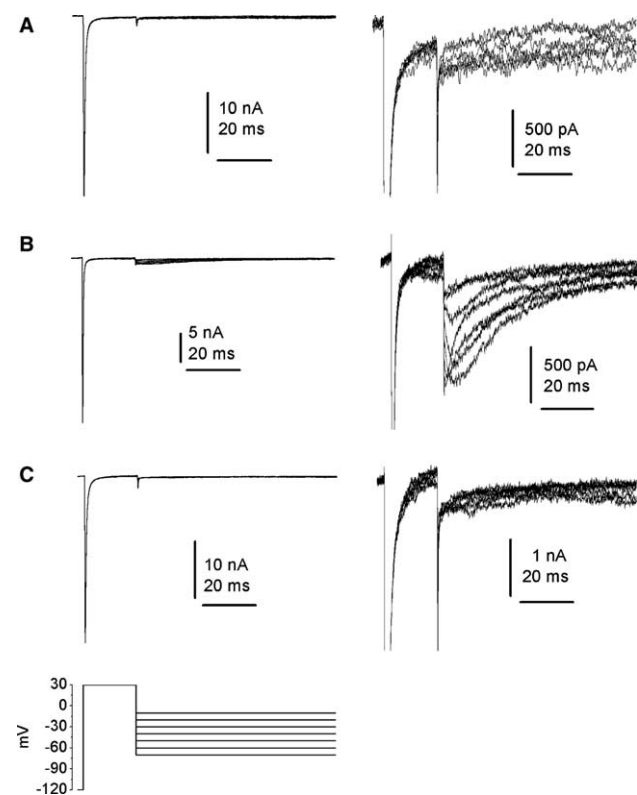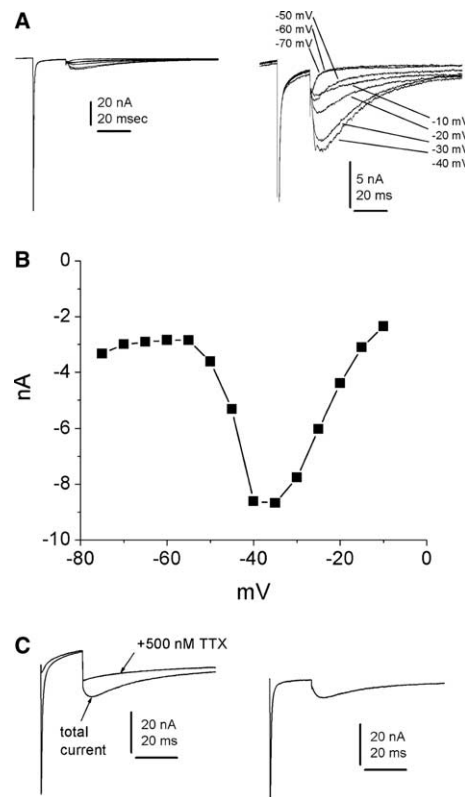

Fig. 1. Resurgent currents are detected in some, but not all, DRG neurons. (A) Family of sodium currents recorded from a small DRG neuron. The peak sodium current amplitude was 49 nA and the cell capacitance was 29 pF. Small neurons did not exhibit resurgent sodium currents in response to repolarization. (B) Sodium currents recorded from an isolated mouse large DRG neuron. The peak sodium current amplitude was 54 nA and the cell capacitance was 37 pF. This cell exhibited large resurgent currents. (C) Representative sodium currents recorded from a large DRG neuron that did not exhibit resurgent currents. The peak sodium current amplitude was 163 nA and the cell capacitance was 43 pF. The voltage protocol used to elicit the currents is shown at the bottom of the figure. Resurgent currents were evoked by repolarizations to voltages ranging from −10 to −70 mV. The amplitudes of the current traces on the right side are magnified 20 times for comparison of resurgent currents.

Fig. 2. TTX-sensitive resurgent currents are generated in Na$_v$1.8-null but not Na$_v$1.6-null DRG neurons. (A) Representative resurgent sodium currents recorded from a large (64 pF) Na$_v$1.8-null DRG neuron. The amplitudes of the current traces on the right side are magnified in order to better see the resurgent currents. (B) The voltage-dependence of the resurgent currents in (A) is shown by plotting the peak resurgent current amplitude against the repolarization pulse potential. (C) The left panel shows currents from a large (62 pF) Na$_v$1.8-null neuron before and after exposure to TTX (500 nM). The right panel shows the TTX-sensitive current (obtained by subtracting the after-TTX current from the before-TTX current). The currents were evoked by a repolarization to −45 mV following a 20 ms depolarization to +30 mV.

3.1.1. DRG neurons from $Na_v1.6$-null mice do not produce resurgent currents. Previous studies have shown that TTX-S resurgent currents are much smaller in cerebellar Purkinje neurons isolated from $Na_v1.6$-null mice than from wild-type mice [9]. We investigated the presence of resurgent currents in DRG neurons from $Na_v1.6$-null mice. Resurgent currents, either TTX-S or TTX-R, were not observed in small ($n = 16$) or large diameter ($n = 25$) $Na_v1.6$-null DRG neurons (data not shown). These results indicate that $Na_v1.6$ underlies the resurgent current in DRG neurons. However, because $Na_v1.6$-null mice are juvenile lethal and may have developmental abnormalities [18], it is also possible that the lack of resurgent currents in DRG neurons from these mice is an indirect consequence of the disruption of $Na_v1.6$ (possibly by altering kinase activity or the expression of beta subunits that could conceivably influence resurgent currents generated by other sodium channel isoforms).

3.1.2. Recombinant $Na_v1.6$ channels generate resurgent currents. To further investigate if $Na_v1.6$ channels generate resurgent currents in DRG neurons, we expressed $Na_v1.6$ channels directly in $Na_v1.8$-null mice DRG neurons using the $Na_v1.6r$ version of this channel [6]. The $Na_v1.8$-null DRG neurons were transfected with GFP alone or in combination with $Na_v1.6r$ cDNA after 5–7 days in culture. Sodium currents were recorded in the presence of 500 nM TTX, 24–48 h after transfection, permitting recording of $Na_v1.6$ currents without contribution of other TTX-S sodium currents in these cells. While $Na_v1.8$-null neurons transfected with GFP alone did not produce fast-inactivating, TTX-R sodium currents, the $Na_v1.6r$ channels produced large fast-inactivating TTX-R sodium currents following biolistic transfection of $Na_v1.8$-null DRG neurons, consistent with our previous findings [6]. Resurgent currents were observed in 62% of the $Na_v1.8$-null DRG neurons ($n = 45$) transfected with $Na_v1.6r$ cDNA (Fig. 3A). In many of these neurons the resurgent currents were quite large, ranging from 0.4% to 6.4% of the peak current

amplitude. Overall, the resurgent current amplitude averaged $1.6 \pm 0.2\%$ of the peak transient sodium current amplitude. However, 38% of the cells expressing $Na_v1.6r$ exhibited no detectable resurgent current (Fig. 3B). It is not clear what contributed to the variation in resurgent current generation. There was no clear correlation either between peak current amplitude and relative amplitude of the resurgent current, or between cell capacitance and the amplitude of the resurgent current. Mean cell capacitance was 24 ± 2 pF (range 8–64 pF). Lidocaine (2 mM) equally inhibited both the resurgent current and peak transient current amplitudes (by $68 \pm 4\%$ and $69 \pm 1\%$, respectively; $n = 3$; holding potential set at -120 mV).

In contrast to $Na_v1.6r$-transfected cells, we did not observe resurgent currents in $Na_v1.8$-null DRG neurons that were biolistically transfected with GFP alone or with a TTX-R version of the rat skeletal muscle sodium channel $Na_v1.4$ ($n = 41$; Fig. 3C). This is despite that fact that $Na_v1.4r$ channels generated larger peak sodium current amplitudes (76 ± 8 nA) than $Na_v1.6r$ channels (40 ± 3 nA).

4. Discussion

We have shown that a subpopulation of DRG neurons generate TTX-sensitive resurgent currents. Resurgent currents were not observed in DRG neurons isolated from $Na_v1.6$-null mice. Recombinant Nav1.6 channels, however, could produce large resurgent currents in cultured $Na_v1.8$-null DRG neurons. Thus, our data demonstrate that the $Na_v1.6$ channel isoform can produce resurgent currents in DRG neurons.

Native resurgent sodium currents have not been previously reported in DRG neurons. We show in this study that resurgent sodium currents can be recorded from $\sim$40% of large diameter DRG neurons. In several of the native DRG cells that we studied the resurgent current was >1000 pA. These
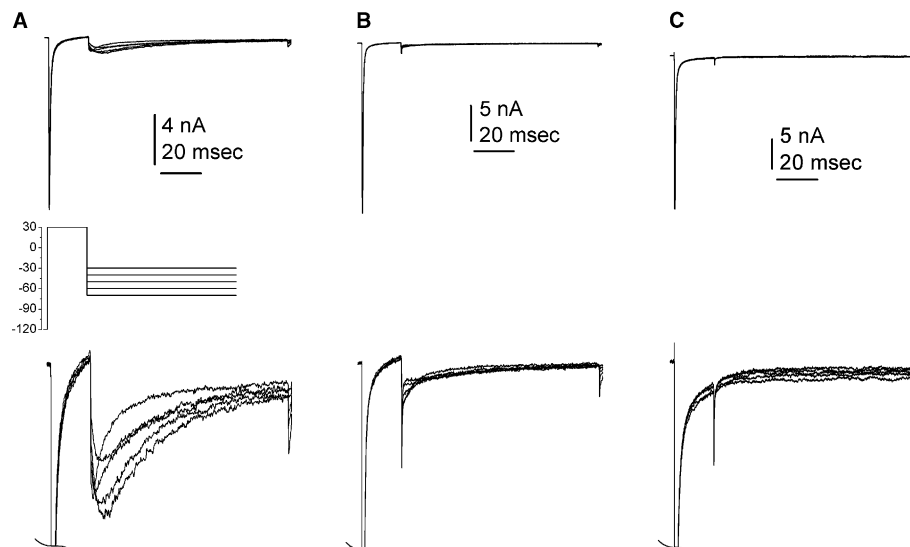

Fig. 3. Resurgent sodium currents generated by recombinant sodium channels. Sodium currents were recorded from $Na_v1.8$-null DRG neurons transfected with $Na_v1.6r$ (A, B) and $Na_v1.4r$ (C) sodium channels. The voltage protocol is shown in (A). Neurons, held at -120 mV, were depolarized to +30 mV for 20 ms, after which they were repolarized to voltages ranging from -30 to -70 mV for 100 ms to elicit resurgent currents. Large resurgent currents were clearly present in some (A) but not all (B) neurons expressing $Na_v1.6r$ currents. (C) Resurgent currents were not detected in neurons expressing $Na_v1.4r$ currents. The lower traces are magnified 10 times for comparison of resurgent currents.

large resurgent currents were similar to those recorded from Purkinje neurons [7], with a clear rising phase and maximum amplitude typically elicited with intermediate repolarizations to voltages close to −40 mV.

Several previous studies have described TTX-S and TTX-R persistent or "late" sodium currents in large [19,20] and small [21,22] DRG neurons. Persistent sodium currents result from channels that fail to completely inactivate, or from window currents that result from overlap in the activation and steady-state inactivation curves. Persistent sodium currents can generate classic tail currents during repolarizations, currents that typically activate instantaneously, decay rapidly and have amplitudes that increase proportionally as the repolarization potential becomes more negative. Although these TTX-R and TTX-S persistent currents can have important influences on excitability, they are distinct from resurgent currents. Resurgent currents exhibit slower activation and decay kinetics during repolarizations than classic tail currents, and have a distinctive voltage-dependence (Fig. 2B). TTX-S "de-inactivating" currents previously observed in large DRG neurons [20] may be related to the resurgent sodium currents that we describe here in large DRG neurons. Small de-inactivating sodium current has also been observed in small DRG neurons [21]. However, because data on these de-inactivating currents are limited and different voltage protocols are used to elicit these currents, it is not clear if these are indeed the same as the resurgent currents that we describe here. It has been proposed that resurgent sodium currents arise from channels that inactivate during strong depolarizations due to the binding of a alternative binding particle [8], possibly the c-terminus of the β4 sodium channel auxiliary subunit [23], that unbinds with a different kinetics from the conventional inactivation particle.

The absence of resurgent currents from DRG neurons isolated from mice lacking functional $Na_v1.6$ channels is consistent with the finding that resurgent currents are reduced by more than 90% in cerebellar Purkinje neurons from $Na_v1.6$-null mice [9]. The small residual resurgent current in Purkinje neurons suggested that other sodium channels can produce these currents. Indeed a recent study indicates that $Na_v1.6$ channels are not the sole generator of resurgent sodium currents in subthalamic nucleus neurons [24]. However, the absence of residual resurgent current in $Na_v1.6$-null DRG neurons strongly suggests that the complement of the TTX-S channels in these neurons, which includes $Na_v1.1$ and $Na_v1.7$ in addition to the TTX-R channels $Na_v1.8$ and $Na_v1.9$, are incapable of producing resurgent currents. Thus, we hypothesize that $Na_v1.2$ can produce a resurgent current. This conclusion is further supported by the absence of $Na_v1.2$, a TTX-S sodium channel that is expressed in Purkinje neurons [3], in adult rodent DRG neurons [25]. In agreement with this view, $Na_v1.2$ can produce a resurgent current when expressed in $Na_v1.8$-null DRG neurons (Rush, Dib-Hajj and Waxman, unpublished data).

Resurgent currents were not observed in small DRG neurons, which have been shown to express $Na_v1.6$ channels [10]. Additionally, resurgent currents have not been recorded from hippocampal CA3 neurons [7] or from spinal neurons [26], even though transcripts of $Na_v1.6$ are detected in these cells. Thus, factors that are differentially expressed in small and large DRG neurons could also be important in determining the ability of large DRG neurons to generate resurgent cur-

rents. For example, the β4 sodium channel auxiliary subunit has been reported to be expressed at higher levels in large DRG neurons than small DRG neurons [27] and a recent report suggests that this subunit might be a crucial modulator of resurgent sodium currents [23]. Phosphorylation has also been implicated in modulating resurgent sodium currents [28] and therefore differential kinase activity might determine which sensory neuron populations express resurgent sodium currents. Cell background appears to be crucial for the production of the resurgent current and, because this current is thought to endow cells with rapid firing, may reflect the functional modality of these sensory neurons.

Irrespective of the mechanisms that regulate resurgent currents, our results demonstrate for the first time that recombinant $Na_v1.6$ channels can produce a resurgent current in DRG neurons. Thus, $Na_v1.6$ sodium channels have the intrinsic capacity to generate resurgent currents. Nevertheless, the competence of different splice variants in generating resurgent currents remains to be investigated. We also show that while some neurons produce a large resurgent current (>3% of the peak current amplitude) other neurons express large fast-inactivating $Na_v1.6$ currents but not detectable resurgent current. Resurgent currents have been shown to play crucial roles in determining the firing patterns of cerebellar Purkinje neurons [29], and therefore it will be important to determine the role that resurgent sodium currents play in sensory neuronal excitability.

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