Membrane properties and electrogenesis in the distal axons of small dorsal root ganglion neurons in vitro

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Vasylyev DV, Waxman SG. Membrane properties and electrogenesis in the distal axons of small dorsal root ganglion neurons in vitro. J Neurophysiol 108: 729–740, 2012. First published May 9, 2012; doi:10.1152/jn.00091.2012.—Although it is generally thought that sensory transduction occurs at or close to peripheral nerve endings, with action potentials subsequently propagating along the axons of dorsal root ganglion (DRG) neurons toward the central nervous system, the small diameter of nociceptive axons and their endings have made it difficult to estimate their membrane properties and electrogenic characteristics. Even the resting potentials of nociceptive axons are unknown. In this study, we developed the capability to record directly with patch-clamp electrodes from the small-diameter distal axons of DRG neurons in vitro. We showed using current-clamp recordings that (1) these sensory axons have a resting potential of −60.2 ± 1 mV; (2) both tetrodotoxin (TTX)-sensitive (TTX-S) and TTX-resistant (TTX-R) Na⁺ channels are present and available for activation at resting potential, at densities that can support action potential electrogenesis in these axons; (3) TTX-sensitive channels contribute to the amplification of small depolarizations that are subthreshold with respect to the action potential in these axons; (4) TTX-R channels can support the production of action potentials in these axons; and (5) these TTX-R channels can produce repetitive firing, even at depolarized membrane potentials where TTX-S channels are inactivated. Finally, using voltage-clamp recordings with an action potential as the command, we confirmed the presence of both TTX-S and TTX-R channels, which are activated sequentially during action potential in these axons. These results provide direct evidence for the presence of TTX-S and TTX-R Na⁺ channels that are functionally available at resting potential and contribute to electrogenesis in small-diameter afferent axons.

sodium channels; tetrodotoxin; resting potential; action potential

IN SENSORY NEURONS, normal transduction occurs in the periphery with action potentials subsequently propagating toward the central nervous system. Moreover, there is evidence suggesting that ectopic impulses that contribute to chronic pain can originate in and propagate along small-diameter afferents within injured or diseased peripheral nerves (Amir and Devor 1993; Meyer et al. 1985; Serra et al. 2011, 2011). However, while substantial information is available concerning membrane properties and ion channel expression within the cell bodies of nociceptive dorsal root ganglion (DRG) neurons (Caffrey et al. 1992; Cummins and Waxman 1997; Kostyuk et al. 1981), the small diameter of the nociceptive axons and free nerve endings that arise from these cells has made it difficult to estimate their membrane properties and electrogenic characteristics.

Pharmacological studies using extracellular and compound action potential recordings have provided evidence for the presence of tetrodotoxin (TTX)-resistant (TTX-R) as well as TTX-sensitive (TTX-S) channels in cutaneous C-fibers (Steffens et al. 2001) and slowly conducting dural afferent fibers (Strassman and Raymond 1999) in the rat and in human C-fibers (Lang et al. 2007; Quasthoff et al. 1995). Using suction electrodes to record extracellular currents, Brock et al. (2001) and Carr et al. (2002) presented evidence suggesting that action potentials can invade the distal endings of some corneal nociceptive nerve fibers and suggested the presence of TTX-R as well as TTX-S Na⁺ channels in these peripheral axons. Intracellular recording and direct evaluation of the contribution of TTX-S and TTX-R currents to action potentials in these axons have not, however, been possible. Even the resting potential of nociceptive axons, a factor that determines the availability of operable Na⁺ channels since they are subject to voltage-dependent inactivation, has been enigmatic. Measurement of resting potential is especially important since threshold tracking has demonstrated that membrane potential influences excitability in C-fibers (Moalem-Taylor et al. 2007). Gap-recording methods have demonstrated that activation of protein kinase depolarizes the resting potential of C-fibers, presumably by opening Na⁺ and Cl⁻ channels (Rang and Ritchie 1988), but these techniques monitor relative changes in membrane potential and have not permitted actual resting potential to be measured. Noting that TTX-R voltage-gated Na⁺ (Naᵥ)1.8 channels activate at relatively positive potentials (about −40 mV) and have a half-maximal voltage for inactivation close to −30 mV, and that extracellular hyperpolarizing currents can increase the amplitude of nerve terminal impulses in corneal fibers, Carr et al. (2002) suggested that resting membrane potential might be lower than −30 mV in these fibers. The small diameter of these axons, however, precluded more direct measurement of their resting potential or other membrane parameters.

Immunocytochemical studies have demonstrated the presence of Naᵥ1.6, Naᵥ1.7, Naᵥ1.8, and Naᵥ1.9 channel isoforms along the trunks of peripheral C-fibers (Black et al. 2002; Black and Waxman 2002; Renganathan et al. 2002; Rush et al. 2005a, 2005b) and have recently demonstrated these Na⁺ channel isoforms within the epidermal free nerve endings of C-fibers (Persson et al. 2010). However, Naᵥ1.6, Naᵥ1.7, and Naᵥ1.9 all inactivate at depolarized membrane potentials and are only available to activate at membrane potentials much lower than the resting potential.
more negative than $-30 \text{ mV}$ (Catterall et al. 2005), underscoring the importance of direct assessment of resting potential and of the contribution of TTX-S and TTX-R Na$^{+}$ channels to electrogenesis in the axons of small DRG neurons. We thus developed the capability to record directly using patch-clamp electrodes from the small-diameter distal axons of small DRG neurons in vitro. Here, we present a current-clamp analysis of electrogenesis and a demonstration by voltage clamp of the presence of both TTX-S and TTX-R currents, and their sequential activation during the action potential, in these small-diameter peripheral nonmyelinated axons.

**METHODS**

*Primary culture of DRG neurons.* DRG neurons from Sprague-Dawley rat pups (*postnatal days 0–5*) were carefully dissected as previously described (Dib-Hajj et al. 2009; Estacion et al. 2008). In brief, isolated ganglia were placed in ice-cold oxygenated complete saline solution (CSS) containing (in mM) 137 NaCl, 5.3 KCl, 1 MgCl$_2$, 25 sorbitol, 3 CaCl$_2$, and 10 HEPES (pH 7.2). Enzymatic treatment was performed in oxygenated, 37°C CSS containing 1.5 mg/ml collagenase A (11088785103, Roche Applied Science) and 0.6 mM EDTA with gentle agitation at 37°C for 20 min after an incubation in oxygenated, 37°C CSS containing 1.5 mg/ml collagenase D (1108874103, Roche Applied Science), 0.6 mM EDTA, and 30 U/ml papain (LS003126, Worthington Biochemicals) with gentle agitation at 37°C for 20 min. The CSS was replaced with DRG media [DMEM-F-12 (11320, Invitrogen) with 100 U/ml penicillin, 0.1 mg/ml streptomycin (15140-122, Invitrogen), 2 mML-glutamine (25030-081, Invitrogen), and 10% FBS (SH30071-02, HyClone) supplemented with 1.5 mg/ml BSA (A9418, Sigma-Aldrich) and 1.5 mg/ml trypsin inhibitor (10109878001, Roche Applied Science)], and the ganglia...
were dispersed by gentle trituration. Cells were plated on poly-d-lysine-coated FluoroDishes (FD35PDL, World Precision Instruments) and maintained at 37°C in a 5% CO2 incubator for 24–32 h in DRG media supplemented with 50 ng/ml each of murine nerve growth factor (Alomone Labs, Jerusalem, Israel) and glial cell-derived neurotrophic factor (Peprotec, Rocky Hill, NJ).

**Electrophysiology.** Distal regions of axons with apparent diameters that varied along the axonal shaft from <0.5 to 1.5 μm were visually identified under a CFI Plan Fluor DII ×100 oil (numerical aperture: 1.3) wdi 16 objective (Nikon, Melville, NY) and tracked back to DRG neuron soma. We recorded from axons that arose from small-diameter (16–30 μm, 24.1 ± 1.1 μm) DRG neurons. Axon endings were current clamped in the whole cell configuration using patch pipettes pulled from glass capillaries (catalog no. PG10165-4, World Precision Instruments,) carefully positioned to form gigaseals with axons using a Burleigh PCS-5000 micromanipulator. The small diameter of the target axons required close attention to physical stability of the pipette seal (strict avoidance of vibration, periodic oscillations in ambient temperature around a set point caused by imperfect room temperature control systems or air movement). Pipette resistance was 5–8 MΩ when filled with intracellular solution, which contained (in mM) 140 KCl, 3 MgATP, 0.5 EGTA, 5 HEPES, and 10 glucose (pH 7.3 with KOH, 325 mosM with sucrose). The pipette solution was not compensated. Data were recorded using an Axopatch 200B amplifier in the fast current-clamp mode, digitized by Digidata 1322A DAC, and stored on the hard disk using pCLAMP 10 software (all from Molecular Devices, Sunnyvale, CA). Voltage traces were filtered at 10 kHz and digitized at 50 kHz. The pipette solution for voltage-clamp recording was (in mM) 140 CsCl, 10 NaCl, 0.5 EGTA, 10 HEPES, 3 Mg-ATP, and 10 glucose (pH adjusted to 7.3 with CsOH, osmolarity adjusted to 330 mosM with sucrose). The series resistance of 17–25 MΩ was compensated by 75%. The extracellular solution contained (in mM) 70 NaCl, 70 choline-Cl, 3 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 20 TEA-Cl, 5 CsCl, and 0.1 CdCl2 (pH adjusted to 7.3 with NaOH, 330 mosM). The junction potential between the pipette and bath solutions was −4.0 mV (calculated in Clampex10) and was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. The extracellular solution contained (in mM) 1.1 CaCl2, 0.0000052 CuSO4, 0.000124 Fe(NO3)3, 0.0015 FeSO4, 0.3 MgCl2, 0.4 MgSO4, 4.2 KCl, 29 NaHCO3, 120.6 NaCl, 0.5 Na2HPO4, 0.45 NaH2PO4, 0.0015 ZnSO4, 0.5 sodium pyruvate, and 17.5 glucose supplemented with 10 mM HEPES (pH 7.3, 325 mosM with sucrose). The −4.0-mV liquid junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Data were recorded using an Axopatch 200B amplifier in the fast current-clamp mode, digitized by Digidata 1322A DAC, and stored on the hard disk using pCLAMP 10 software (all from Molecular Devices, Sunnyvale, CA). Voltage traces were filtered at 10 kHz and digitized at 50 kHz. The pipette solution for voltage-clamp recording was (in mM) 140 CsCl, 10 NaCl, 0.5 EGTA, 10 HEPES, 3 Mg-ATP, and 10 glucose (pH adjusted to 7.3 with CsOH, osmolarity adjusted to 330 mosM with sucrose). The series resistance of 17–25 MΩ was compensated by 75%. The extracellular solution contained (in mM) 70 NaCl, 70 choline-Cl, 3 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 20 TEA-Cl, 5 CsCl, and 0.1 CdCl2 (pH adjusted to 7.3 with NaOH, 330 mosM). The junction potential between the pipette and bath solutions was −4.0 mV (calculated in Clampex10) and was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated. Voltage-clamp junction potential (calculated in Clampex10) between pipette and bath solutions was not compensated.

**Table 1.** Action potential parameters and passive properties of axons of small dorsal root ganglia neurons.

<table>
<thead>
<tr>
<th>Action Potential Parameter</th>
<th>Axons</th>
<th>Soma</th>
</tr>
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<tbody>
<tr>
<td>Input resistance, MΩ</td>
<td>1.138 ± 97</td>
<td>303 ± 37†</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>−60.2 ± 1.0</td>
<td>−63.0 ± 1.6†</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−29.8 ± 1.3</td>
<td>−19.5 ± 0.5‡</td>
</tr>
<tr>
<td>Threshold, pA</td>
<td>51.2 ± 3.9</td>
<td>425.0 ± 69.9†</td>
</tr>
<tr>
<td>Overshoot, mV</td>
<td>45.9 ± 1.8</td>
<td>65.1 ± 0.7†</td>
</tr>
<tr>
<td>Undershoot, mV</td>
<td>−58.9 ± 1.8</td>
<td>−58.0 ± 1.5</td>
</tr>
<tr>
<td>Maximum rise slope, mV/ms</td>
<td>98.0 ± 8.7</td>
<td>142.4 ± 8.3‡</td>
</tr>
<tr>
<td>Maximum decay slope, mV/ms</td>
<td>−41.7 ± 2.9</td>
<td>−48.3 ± 3.4</td>
</tr>
<tr>
<td>Membrane potential at maximum rise slope, mV</td>
<td>19.9 ± 1.4</td>
<td>39.7 ± 1.0‡</td>
</tr>
<tr>
<td>Membrane potential at maximum decay slope, mV</td>
<td>27.7 ± 2.0</td>
<td>42.3 ± 1.8*</td>
</tr>
<tr>
<td>Threshold, ms</td>
<td>6.5 ± 1.0</td>
<td>10.9 ± 2.5</td>
</tr>
<tr>
<td>Rise time, ms</td>
<td>4.0 ± 0.3</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Decay time, ms</td>
<td>2.9 ± 0.2</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>Half-width, ms</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Width at 0 mV, ms</td>
<td>2.5 ± 0.2</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Maximum rise slope, ms</td>
<td>3.5 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Maximum decay slope, ms</td>
<td>4.7 ± 0.3</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Undershoot, ms</td>
<td>11.1 ± 0.6</td>
<td>9.5 ± 1.2</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 21 axons and 6 soma. All recordings were made at resting membrane potential (i = 0 holding current). Presented data are from action potentials evoked by 200 ms that were just suprathreshold (5–10 pA above threshold). Half-width was calculated as width at half-amplitude measured from threshold to overshoot. Somatic recordings were performed under the same experimental conditions, but the pipette resistance was 2–2.5 MΩ. Levels of statistical significance of the difference between two means are as follows: *P < 0.01 and †P < 0.001.
(recordings were performed using same equipment and software used for current-clamp recordings. Current traces were filtered at 2–5 kHz and digitized at 50 kHz. The -P6 protocol was used to subtract uncompensated membrane capacitance and leak currents. Recordings were made at room temperature (23–24°C). Data were analyzed using pCLAMP 10 (Molecular Devices), Microsoft Excel (Microsoft, Redmond, WA), and Origin 8.5 (OriginLab, Northampton, MA) software.

The voltage threshold of action potential generation was determined from the inflection point on the rising phase of action potential. The inflection point was determined from the respective extremum of the action potential derivative (dV/dt). The overshoot and undershoot were determined as the maximal and minimal voltage, respectively, on the action potential waveform past voltage threshold. All data are presented as means ± SE. The hypothesis that the population means are significantly different was checked using a Student’s t-test. P values of <0.05 were considered as significant.

RESULTS

Resting membrane potential is close to −60 mV in distal axons of small DRG neurons. Previous extracellular recordings have been interpreted as suggesting that resting membrane potential of the primary afferent nerve terminal region of corneal nociceptors may be depolarized to the extent that TTX-S Na⁺ channels are inactivated (Carr et al. 2002), but direct measurements of resting potential in small-diameter afferents have not been available. The axons assessed in the present study arose from small DRG neurons (see METHODS). In this study, we obtained patch-clamp recordings in the whole cell mode and measured the resting membrane potential of distal axons with apparent diameter that varied along the axonal shaft between <0.5 and 0.8 μm over most of the axon trajectory. For the axons studied, the most distal 20–40 μm of the shaft immediately proximal to the axon ending tended to exceed 0.8 μm in diameter, with the tip of the axon ending (~5-μm length) forming an expansion of 1.5- to 3-μm width. Along the remaining axon shaft the diameter was <0.8 μm for 85–90% of the axon trajectory, with diameter exceeding 0.8 μm for <10–15% of the total axon length and only rarely exceeding 1.0 μm (Fig. 1). Axon input resistance was relatively high, ranging from 310 to 1,846 MΩ (1.138 ± 97 MΩ, n = 21). The recording site was several length constants away from the DRG neuron soma to ensure electrotonic isolation (Fig. 1). None of the distal axons studied gave rise to terminals that contacted other neurons.

Resting membrane potential ranged from −68 to −53 mV (−60.2 ± 1.0 mV, n = 21) in these axons when measured within 30 s after the whole cell configuration was established (Fig. 2). Action potentials were evoked 5 min later, when resting membrane potential stabilized at −59.1 ± 0.8 mV (range: −64 to −51 mV, n = 21), which was not significantly different from resting membrane potential measured at the 30-s time point (n = 21, P > 0.05). These measurements of resting potential are consistent with previously reported values of resting membrane potential along the shafts of myelinated axons within rat sciatic nerve obtained using sharp microelectrodes for intra-axonal recordings (Kocsis et al. 1982, 1983).

Distal parts of small DRG neuron axons generate all-or-none action potentials at a wide range of baseline membrane potentials. Distal axons generated overshooting action potentials of 2.5 ± 0.2-ms duration (calculated at 0 mV) in response to depolarizing current injections (Fig. 3). The current threshold varied from 20 to 90 pA (51.2 ± 3.9 pA, n = 21). Action potential voltage threshold, determined from the inflection point on the membrane potential rising phase identified from its first differential dV/dt, ranged from −14.2 to −42.6 mV (−29.8 ± 1.3 mV, n = 21). The overshoot was 45.9 ± 1.8 mV, whereas the undershoot was −58.9 ± 1.8 mV (n = 21). The standard action potential pa-

![Fig. 4. Distal axons of small DRG neurons generate action potential in an all-or-none fashion at a wide range of membrane potentials. A–C: action potentials recorded from the same distal axon held at different baseline membrane voltages (−40 mV (A), −60 mV (B), and −80 mV (C)) by steady-state current injections. Action potentials were elicited by 200-ms depolarizing current injections ranging from 0 to 60 pA in 10-pA steps (A) or from 0 to 120 pA in 10-pA steps (B and C). The threshold current was 25 pA (A), 90 pA (B), and 95 pA (C). The threshold current evoked an action potential with the following overshoots: 42 mV (A), 37 mV (B), and 36 mV (C) and undershoots: −51 mV (A), −56 mV (B), and −68 mV (C).](http://jn.physiology.org/)

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Parameters of distal axons evoked from native resting potential (−60.2 ± 1.0 mV, n = 21) are shown in Table 1.

To determine whether Na,1.8 channels can support action potentials in these axons, we injected depolarizing current that reduced membrane potential to −40 mV, a potential at which the other Na⁺ channels functionally expressed in small DRG neurons, with the exception of the Na,1.8 isoform, are inactivated (Cummins et al. 2007; Cummins and Waxman 1997). Overshooting action potentials still could be evoked from the −40-mV baseline membrane potential (Fig. 4A). Moreover, 10 of 11 axon terminals studied using this protocol were able to generate repetitive firing during 200-ms depolarizing test current pulses when evoked from the −40-mV baseline potential. Compared with action potentials evoked from the −60-mV baseline potential, action potentials evoked from the −40-mV baseline membrane potential displayed reduced current threshold (29.1 ± 2.4 pA at −40 mV and 46.4 ± 6.2 pA at −60 mV).

Fig. 5. The action potential of an axon of a small DRG neuron has inflections on the falling phase. The action potential is shown overlaid on its first differential, which shows the rate of change of the membrane potential (dV/dt). Action potentials were recorded from the same distal axon and were evoked at resting membrane potential (B) and at baseline membrane potential adjusted to −40 mV (A) or −80 mV (C) by injection of depolarizing (A) or hyperpolarizing (B) holding current. The presented action potentials were evoked by 200-ms depolarizing current injections 5 pA above the threshold: 40 pA (A), 75 pA (B), and 90 pA (C). Note the presence of two peaks in the dV/dt trace on the repolarizing phase of the action potential. The shoulder on the falling phase of the action potential (indicated by the arrowhead) was determined from the extremum (local maximum) of dV/dt.

Fig. 6. Tetrodotoxin (TTX)-sensitive (TTX-S) Na⁺ channels contribute to subthreshold properties of distal axons of small DRG neurons. A: subthreshold membrane responses evoked by 200 ms of 30-pA (bottom), 35-pA (middle), and 75-pA (top) depolarizing current injections in control solution (solid line) and after the application of 300 nM TTX (dotted line). The value for subthreshold overshoot was 14.1 mV (top), 7.3 mV (middle), and 1.7 mV (bottom) in control solution and 6.7 mV (top), 1.1 mV (middle), and −0.2 mV (bottom) in 300 nM TTX. Note the presence of the TTX-S component of active voltage amplification, which activated at around −50 mV. B: membrane voltage overshoot plotted against stimulation current in control solution (●) and in 300 nM TTX (○). The voltage overshoot was determined as the difference between peak voltage at the rising phase of the membrane potential and the plateau voltage 20–40 ms after the peak (shown by arrows). Data are means ± SE; n = 4–6. *P < 0.05 for the level of statistical significance of the difference between two means.
$n = 11, P < 0.05$), depolarized voltage threshold ($-18.7 \pm 1.7$ mV at $-40$ mV and $-30.3 \pm 1.1$ at $-60$ mV, $n = 11, P < 0.001$), increased action potential duration at 0 mV ($4.3 \pm 0.5$ ms at $-40$ mV and $2.4 \pm 0.2$ ms at $-60$ mV, $n = 11, P < 0.01$), decreased action potential maximal rise slope ($55.5 \pm 7.8$ mV/ms at $-40$ mV and $90.9 \pm 9.3$ mV/ms at $-60$ mV, $n = 11, P < 0.01$), decreased absolute value of action potential maximal decay slope ($-18.2 \pm 5.8$ mV/ms at $-40$ mV and $-41.1 \pm 3.5$ mV/ms at $-60$ mV, $n = 11, P < 0.01$), and depolarized undershoot ($-40.3 \pm 3.2$ mV at $-40$ mV and $-60.1 \pm 2.5$ at $-60$ mV, $n = 11, P < 0.001$), but did not display significantly different action potential overshoot ($42.8 \pm 2.3$ mV at $-80$ mV and $45.8 \pm 2.3$ mV at $-60$ mV, $n = 11, P > 0.05$; Fig. 4). Hyperpolarization of the membrane potential to $-80$ mV resulted in increased current threshold, which, however, did not reach the level of statistical significance ($60.0 \pm 5.8$ pA at $-80$ mV and $46.4 \pm 6.2$ pA at $-60$ mV, $n = 11, P > 0.05$), hyperpolarized voltage threshold ($-42.0 \pm 1.4$ mV at $-80$ mV and $-30.3 \pm 1.1$ at $-60$ mV, $n = 11, P < 0.001$), decreased action potential duration at 0 mV ($1.7 \pm 0.2$ ms at $-80$ mV and $2.4 \pm 0.2$ ms at $-60$ mV, $n = 11, P < 0.05$), increased action potential maximal rise slope ($135.8 \pm 13.7$ mV/ms at $-80$ mV and $90.9 \pm 9.3$ mV/ms at $-60$ mV, $n = 11, P < 0.05$), increased absolute value of action potential maximal decay slope ($-53.9 \pm 3.9$ mV/ms at $-80$ mV and $-41.1 \pm 3.5$ mV/ms at $-60$ mV, $n = 11, P < 0.05$), and hyperpolarized undershoot ($-67.8 \pm 2.3$ mV at $-80$ mV and $-60.1 \pm 2.5$ at $-60$ mV, $n = 11, P < 0.05$), but did not significantly affect action potential overshoot ($42.8 \pm 2.3$ mV at $-80$ mV and $45.8 \pm 2.3$ mV at $-60$ mV, $n = 11, P > 0.05$; Fig. 4C). These results show that, while small-diameter DRG axons have resting potentials of about $-60$ mV, they are capable of generating action potentials even when depolarized to voltages where only Na1.8 channels are available. Our observations demonstrate, in fact, reduced current threshold for action potential generation at $-40$ mV, consistent with patch-clamp experiments (Harty et al. 2006; Snape et al. 2010) and modeling results (Choi and Waxman 2011) for DRG neuron cell bodies.

The action potential of distal axons has inflections on the falling phase. The presence of inflections (a shoulder) on the repolarizing phase of the action potential has been repeatedly observed in DRG neurons and can be clearly shown by demonstration of two peaks on the falling phase of $dV/dt$ (Renganathan et al. 2001). We found that, in 12 of 21 distal axon regions examined, there were inflections on the falling phase of the action potential when evoked from resting membrane potential (Fig. 5B). We further studied action potential config-

![Image](http://jn.physiology.org/)

Fig. 7. Distal axons can generate TTX-resistant (TTX-R) action potentials. A and B: action potentials were generated by 0-, 10-, 20-, 30-, 40-, and 50-pA current injections in control solution (A) and after the application of 300 nM TTX (B). As shown from the traces, TTX increased the current threshold of action potential initiation. C and D (same distal axon as in A and B): membrane potential was held at $-40$ mV by an injection of sustained depolarizing current. Action potentials were generated by 20- and 40-pA current injections. Note that TTX did not significantly affect repetitive firing.
uration in a subgroup (11 of 21) of axons at resting potential, at −40-mV holding potential, and at −80-mV holding potential: 8 of these 11 distal axons had inflections on the falling phase of action potential when evoked from the −40-mV holding potential (Fig. 5, A and B). Action potentials could be evoked from all of the 11 studied axons from −80 mV, and when action potentials were evoked from −80 mV, these two peaks on the action potential derivative became more pronounced (Fig. 5C).

TTX-S Na\(^+\) channels contribute to subthreshold membrane properties of distal axons. TTX-S Na\(_{1.7}\) channels expressed in small DRG neurons activate at relatively hyperpolarized membrane potentials, contributing to the amplification of subthreshold depolarizations (Cummins et al. 1998; Herzog et al. 2003). To determine whether low-threshold TTX-S Na\(^+\) channels are functionally expressed in distal axons, we measured the membrane response to small (10–45 pA) depolarizing currents, which did not reach threshold for action potential generation, applied with 5-pA increments. Subthreshold amplification of the passive membrane depolarization began to develop for responses that depolarized the membrane to about −55 mV, a voltage consistent with activation of Na\(_{1.7}\) channels (Cummins et al. 1998; Herzog et al. 2003), and was attenuated by exposure to 300 nM TTX (Fig. 6A). The voltage overshoot of the amplified response was determined as the difference between peak voltage at the rising phase of the membrane potential and the plateau voltage, measured 20–40 ms after the peak, as shown between the arrows in Fig. 6A. Figure 6B shows the amplitude of the voltage overshoot before and after exposure to 300 nM TTX at a range of stimulus intensities. At 30- to 45-pA stimulation current, the amplitude of the voltage overshoot was reduced by 55–75% by 300 nM TTX (Fig. 6B). These results establish that TTX-S Na\(^+\) channels can contribute to subthreshold membrane properties of the distal axons of small DRG neurons.

Both TTX-S and TTX-R Na\(^+\) channels contribute to the generation of action potentials in the axons of DRG neurons. Action potentials with inflections in C-type DRG neurons have been shown to persist in the presence of TTX (Harper and Lawson 1985a, 1985b; Morita and Katayama 1989; Ritter and Mendell 1992), a property largely determined by the presence of the TTX-R Na\(_{1.8}\) channel (Kostyuk et al. 1981; Renganathan et al. 2001; Waddell and Lawson 1990). Although application of 300 nM TTX did not abolish action potential generation in any of the axons studied (n = 6), its effect on action potential electrogenesis was significant. At a membrane potential of −60 mV, TTX depolarized voltage threshold by 8.1 mV (from −31.9 ± 2.0 mV in control to −23.8 ± 2.4 mV in 300 nM TTX, n = 6, P < 0.05) and increased current threshold by 81% (38.3 ± 7.1 pA in control and 69.2 ± 11.5 pA in the presence of TTX, n = 6, P < 0.05; Figs. 7, A and B, and 8, A and C). In contrast, when membrane potential was depolarized to −40 mV, where TTX-S fast Na\(^+\) channels expressed in DRG neurons are inactivated but TTX-R Na\(_{1.8}\) channels are still available for activation, TTX did not significantly affect the voltage threshold (−22.2 ± 1.8 mV in control and −18.6 ± 1.6 mV in TTX, n = 6, P > 0.05) or the current threshold (25.8 ± 3.5 pA in control and 30 ± 7.6 pA in TTX, n = 6, P > 0.05; Figs. 7, C and D, and 8, B and D). Repetitive firing was present in axons stimulated at −40 mV after exposure either to 300 nM TTX (Fig. 7, C and D) or 100 μM CdCl\(_2\) (Fig. 9), consistent with previous findings (Blair and Bean 2003, 2002; Renganathan et al. 2001) showing that Na\(_{1.8}\) can support repetitive firing in response to sustained depolarization. Inhibition of Ca\(^{2+}\) channels by 100 μM CdCl\(_2\) significantly attenuated the peak amplitude of the first extremum of dV/dt, which reflects a reduction of the action potential maximum decay slope. However, it did not abolish the inflection point on the falling phase of the action potential (Fig. 10), suggesting a substantial contribution of Na\(^+\) channels to the production of the shoulder on the falling phase of the action potential.

We further studied the contribution of TTS-S and TTX-R Na\(^+\) channels to action potential electrogenesis by recording axonal Na\(^+\) currents evoked by a voltage command shaped in the form of the action potential previously recorded from a different axon ending (Fig. 11A, bottom). Both TTS-S and TTX-R Na\(^+\) currents were present in the distal axons (Fig. 11A, bottom). The peak Na\(^+\) current was 176.4 ± 27.8 pA (n = 4); the amplitudes of TTS-S and TTX-R currents were 136.6 ± 21.2 pA (n = 4) and 68.6 ± 29.5 pA (n = 4), respectively (Fig. 11B). Application of 10 mM lidocaine blocked the TTX-R
current by 89%, as expected for a TTX-R Na\(^+\) current (n = 2; Fig. 11B). Thus, both TTX-S and TTX-R Na\(^+\) channels contribute to action potential electrogenesis in the distal axons of cultured small DRG neurons, although the contribution of TTX-S channels is reduced by depolarization. The effect of TTX on action potential parameters is shown in Table 2.

**DISCUSSION**

Information about the functional properties of the fine caliber axons of small DRG neurons, which include nociceptors (Harper and Lawson 1985a, 1985b), is especially important since sensory transduction occurs distally within these axons or their endings and ectopic impulses that contribute to neuropathic pain can be generated at these sites (Amir and Devor 1993; Meyer et al. 1985; Serra et al. 2012; Serra et al. 2011). While substantial information about the passive and active membrane properties of the cell bodies of small-diameter DRG neurons has been obtained from voltage clamp (Caffrey et al. 1992; Cummins and Waxman 1997; Elliott and Elliott 1993; Kostyuk et al. 1981) and current clamp (Caffrey et al. 1992; Cummins et al. 2009; Dib-Hajj et al. 2005; Faber et al. 2011; Renganathan et al. 2001), much less is known about membrane properties and electrogenesis within the peripheral axons of these cells, particularly the fine caliber (<1 \(\mu\)m) axons of small DRG neurons. Compound action potential studies have suggested that both TTX-R and TTX-S channels contribute to the conduction of action potentials along mammalian peripheral nonmyelinated C-fibers (Quasthoff et al. 1995; Steffens et al. 2001), and immunocytochemical studies have demonstrated the presence of Na\(_v\)1.6, Na\(_v\)1.7, Na\(_v\)1.8 and Na\(_v\)1.9 channels along the trunks of C-fibers (Black et al. 2002; Black and Waxman 2002; Renganathan et al. 2002; Rush et al. 2005a, 2005b). Persson et al. (2010) have demonstrated the presence of immunoreactivity for TTX-sensitive Na\(^+\) channels Na\(_v\)1.6 and Na\(_v\)1.7 and TTX-resistant Na\(^+\) channels Na\(_v\)1.8 and Na\(_v\)1.9 within epithelial free nerve endings, which are the distal tips of small-diameter peripheral nociceptive nerve fibers. The voltage dependences of activation and steady-state inactivation for each of these channels are different (Catterall et al. 2005; Cummins et al. 2007; Rush et al. 2005a, 2005b), with Na\(_v\)1.8 displaying steady-state inactivation with voltage dependence that is depolarized by ~30 mV with respect to the other channels (Akopian et al. 1996; Cummins and Waxman 1997; Hudmon et al. 2008). To date, however, the question of whether the resting potential of small-diameter peripheral axons of DRG neurons is similar to that in DRG neuron cell bodies has remained open. Carr et al. (2002) inferred that TTX-R channels, most likely Na\(_v\)1.8, are present at densities

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**Fig. 9.** Inhibition of Ca\(^{2+}\) channels did not block action potential generation or repetitive firing. A and B: representative recordings (n = 4) of action potentials evoked by 0-, 10-, 20-, 30-, 40-, 50-, and 60-pA current injections in control solution (A) and after the application of 100 \(\mu\)M CdCl\(_2\) (B). C and D: membrane potential was held at −40 mV by an injection of sustained depolarizing current. Action potentials were generated by 30- and 40-pA current injections.
that could support action potential generation within the distal regions of corneal nerve endings and observed that hyperpolarization increases the amplitude of extracellularly recorded nerve impulses within these endings, a finding that they interpreted as suggesting a resting potential lower than −30 mV, but they based these suggestions on extracellular recordings.

In this study, we recorded using patch-clamp electrodes and demonstrated a contribution of TTX-R as well as TTX-S Na channels to electrogenesis in the distal axons of small-diameter DRG neurons in vitro. Using patch-clamp electrodes to record in current-clamp mode, we recorded from the axons at sites 400–700 μm from the cell body. The membrane resistivity, calculated from axon input resistance and axon membrane capacitance (5.5 ± 0.5 pF, n = 20, estimated by integrating the capacitive transient current due to −10-mV steps from a −60-mV holding potential), was 6.2 ± 0.8 kΩ·cm−2 (n = 20).

The length constant was calculated based on the following equation: λ = (d × Rm/4Rt)1/2, where λ is the length constant, d is axonal diameter, Rm is axonal membrane resistivity, and Rt is resistivity of the axoplasm. Assuming a model with uniform axon diameter of 0.6 μm (Fig. 1B), a resistivity of axoplasm of 200 Ω·cm (Jamieson et al. 2003; Rall et al. 1992), and membrane capacitance of 1 μF/cm2, we estimate that these axons have an average length constant of 207 ± 13 μm (n = 20). While the axons under study displayed some variation in diameter along their length, diameter was often 0.5 μm or less and rarely exceeded 0.8 μm. The average diameter of the axon shown in Fig. 1B was estimated to be 0.7 μm, if all axonal regions were averaged, or 0.6 μm, if the first 30-μm segment immediately proximal to the axon ending was not included (estimation was performed by subdividing the 720-μm axon into smaller parts of equal lengths and then averaging their diameters). Increasing the axon diameter from 0.6 to 0.8 μm would result in a 15% increase of the average length constant in our model, which would not significantly affect our estimation of the electrotonic separation between the soma and the recording site. Thus, we conclude that our recordings were obtained at two to four length constants from the cell body.

minimizing the contribution of passive or active membrane properties of the cell body to the results that we obtained.

Our recordings used a pipette solution similar to that used previously in current-clamp recordings from the somata of small DRG neurons (Cummins et al. 2009; Dib-Hajj et al. 2005; Faber et al. 2011; Renganathan et al. 2001). Since resting potential is dependent on the transmembrane K+ gradient, we cannot exclude an experimental error due to differences that might exist between intracellular K+ concentrations in cell bodies versus axons and in diffusion between the pipette and small volume axon in the present study compared with large volume cell bodies in earlier studies. Nevertheless, we believe that our recordings produce an acceptable measure of resting potential. Under our recording conditions, distal axons displayed a resting potential of −60.2 ± 1.0 mV, a value that is close to the resting potential of the soma of small DRG neurons (−63.0 ± 1.6 mV) recorded under the same conditions. Even assuming an experimental error of 5 mV in the hyperpolarizing direction, our results suggest that resting potential in distal sensory axons is not substantially different from that in cell bodies. Assuming that our measurements accurately represent the values in small diameter axons, the following values represent a comparison that can be drawn between distal axon action potential parameters and action potential parameters of somata of small DRG neurons recorded from the same culture under the same conditions (Table 1): current threshold, 51.2 pA (425 pA); voltage threshold, −29.8 mV (−19.5 mV); overshoot, 45.9 mV (65.1 mV); maximal rise slope, 98 mV/ms (142.4 mV/ms); maximal decay slope, −41.7 mV/ms (−48.3 mV/ms); rise time, 4 ms (3.2 ms); decay time, 2.9 ms (2.8 ms); width at 0 mV, 2.5 ms (2.8 ms); and half-width, 2 ms (1.7 ms), for distal axons (DRG neuron somas), respectively.

We would emphasize that our observations have been made in cultured neurons and do not delineate the molecular identities or densities of the Na+ channels within their axons. Low densities of Na+ channels can support action potential electogenesis in small-diameter axons as a result of their high input resistance (Donnelly 2008; Waxman et al. 1989). Nevertheless, taken together with our current-clamp results, our voltage-clamp observations indicate that TTX-R and TTX-S Na+ channels are present and available for activation at resting potential and are activated during action potential electogenesis in the fibers we studied with a time course similar to that described by Blair and Bean (2002) for DRG neuron cell bodies. Our results suggest that TTX-R Na+ channels can support action potential electogenesis in these axons, a finding that we interpret as indicating the presence of Na1.8, given the more rapid activation kinetics of Na1.8 compared with Na1.9, which is also a TTX-R channel (Cummins et al. 1999).

Consistent with this interpretation, patch-clamp studies on the somata of small DRG neurons have demonstrated that, at that site, Na1.8 channels contribute to 60–80% of the inward current underlying the action potential upstroke (Blair and Bean 2002; Renganathan et al. 2001). Our results show that inflections on the falling phase of the action potential in small distal axons persist at −40 mV, suggesting a contribution from Na1.8 channels. Moreover, Cd2+ did not abolish the inflection on the falling phase of the action potential, leading us to conclude that, as suggested by Renganathan et al. (2001), Na1.8 channels contribute substantially to the shoulder on falling phase of the action potential.
While not ruling out a contribution of Ca\(^{2+}\) channels to action potential electrogenesis, our observations indicate that Na\(^{+}\) channels play a major role in electrogenesis in small-diameter distal axons. Our current-clamp recordings indicate that TTX-R channels can support repetitive firing within the axons of DRG neurons, even at depolarized membrane potentials where TTX-S channels are inactivated. Our results also show that TTX-S Na\(^{+}\) channels contribute to subthreshold membrane properties of distal axons. Although the small size of the current and limited time for stable recording did not permit a quantitative voltage-clamp analysis, we obtained voltage-clamp recordings using a recorded action potential waveform as the command and recorded both TTX-S and TTX-R currents in these axon endings, with an overall signature similar to that for TTX-S and TTX-R currents in DRG neuron cell bodies by Blair and Bean (2002). These results are consistent with the demonstration of Na\(_{a1.6}\) and Na\(_{a1.7}\) immunoreactivity in free nerve endings (Persson et al. 2010) and electrophysiological observations that indicate that these Na\(^{+}\) channel isoforms can amplify small, slow depolarizations that are subthreshold with respect to the action potential (Cummins et al. 1998; Herzog et al. 2003; Rush et al. 2007).

The presence of both Na\(_{a1.7}\) and Na\(_{a1.8}\), at resting potentials where both channels are available for activation, suggests that, as has been demonstrated using similar recording methods to record from the cell bodies of small DRG neurons, these channels can interact physiologically within the axons of small DRG neurons, with Na\(_{a1.7}\) contributing to the amplification of small depolarizing stimuli to bring the membrane to threshold for activation of Na\(_{a1.8}\), which contributes a majority of the current supporting repetitive action potential firing (Rush et al. 2006, 2007). Gain-of-function mutations of Na\(_{a1.7}\) have been shown to produce hyperexcitability of DRG neurons that contain Na\(_{a1.8}\) (Rush et al. 2006), thereby producing pain in inherited erythromelalgia, paroxysmal extreme pain disorder, and painful small fiber neuropathies (Dib-Hajj et al. 2010; Faber et al. 2011). On the basis of the present results we suggest that, if C-fiber properties in vivo are similar to those we have described in vitro, Na\(_{a1.7}\) channels are functionally operable in these small axons, so that mutations of Na\(_{a1.7}\) may produce hyperexcitability not just in the cell bodies of nociceptive DRG neurons in these disorders but also in their axons.

Table 2. Effect of 300 nM TTX on action potential parameters of axons of small dorsal root ganglia neurons

<table>
<thead>
<tr>
<th>Action Potential Parameter</th>
<th>Control</th>
<th>300 nM TTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input resistance, MΩ</td>
<td>1.314 ± 171</td>
<td>ND</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>−61.3 ± 2.2</td>
<td>ND</td>
</tr>
<tr>
<td>Threshold, pA</td>
<td>38.3 ± 7.1</td>
<td>69.2 ± 11.5*</td>
</tr>
<tr>
<td>Overshoot, mV</td>
<td>45.2 ± 3.6</td>
<td>32.9 ± 3.8*</td>
</tr>
<tr>
<td>Undershoot, mV</td>
<td>−63.4 ± 2.8</td>
<td>−50.1 ± 3.5*</td>
</tr>
<tr>
<td>Maximum rise slope, mV/ms</td>
<td>93.5 ± 8.7</td>
<td>51.3 ± 7.9†</td>
</tr>
<tr>
<td>Maximum decay slope, mV/ms</td>
<td>−42.3 ± 4.8</td>
<td>−24.5 ± 2.9†</td>
</tr>
<tr>
<td>Membrane potential at maximum rise slope, mV</td>
<td>19.4 ± 3.6</td>
<td>13.6 ± 3.1</td>
</tr>
<tr>
<td>Membrane potential at maximum decay slope, mV</td>
<td>26.9 ± 3.5</td>
<td>17.7 ± 3.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>300 nM TTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold, ms</td>
<td>7.5 ± 1.6</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>Rise time, ms</td>
<td>4.5 ± 0.7</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Decay time, ms</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Half-width, ms</td>
<td>1.9 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Width at 0 mV, ms</td>
<td>2.3 ± 0.3</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>Maximum rise slope, ms</td>
<td>4.0 ± 0.7</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Maximum decay slope, ms</td>
<td>5.2 ± 0.7</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>Undershoot, ms</td>
<td>12.1 ± 1.4</td>
<td>11.1 ± 0.7</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6. The membrane potential of distal axons was held at −60 mV by an injection of steady-state currents. Presented data are from action potentials evoked by 200-ms suprathreshold current pulses (threshold plus 5–10 pA). TTX, tetrodotoxin; ND, not determined. Levels of statistical significance of the difference between two means are as follows: *P < 0.05 and †P < 0.01.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.V.V. performed experiments; D.V.V. analyzed data; D.V.V. and S.G.W. interpreted results of experiments; D.V.V. prepared figures; D.V.V. and S.G.W. drafted manuscript; S.G.W. conception and design of research; S.G.W. edited and revised manuscript; S.G.W. approved final version of manuscript.

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