Painful Peripheral Nerve Injury Decreases Calcium Current in Axotomized Sensory Neurons

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Background: Reports of Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) loss after injury to peripheral sensory neurons do not discriminate between axotomized and spared neurons. The spinal nerve ligation model separates axotomized from spared neurons innervating the same site. The authors hypothesized that I\(_{\text{Ca}}\) loss is a result of neuronal injury, so they compared axotomized L5 dorsal root ganglion neurons to spared L4 neurons, as well as neurons from rats undergoing skin incision alone.

Methods: After behavioral testing, dissociated neurons from L4 and L5 dorsal root ganglia were studied in both current and voltage patch clamp modes. The biophysical consequence of I\(_{\text{Ca}}\) loss on the action potential was confirmed using selective I\(_{\text{Ca}}\) antagonists. Data were grouped into small, medium, and large cells for comparison.

Results: Reduced I\(_{\text{Ca}}\) was predominantly a consequence of axotomy (L5 after spinal nerve ligation) and was most evident in small and medium neurons. I\(_{\text{Ca}}\) losses were associated with action potential prolongation in small and medium cells, whereas the amplitude and duration of after hyperpolarization was reduced in medium and large neurons. Blockade with Ca\(^{2+}\) channel antagonists showed that action potential prolongation and after hyperpolarization diminution were alike, attributable to the loss of I\(_{\text{Ca}}\).

Conclusion: Axotomy is required for I\(_{\text{Ca}}\) loss. I\(_{\text{Ca}}\) loss correlated with changes in the biophysical properties of sensory neuron membranes during action potential generation, which were due to I\(_{\text{Ca}}\) loss leading to decreased outward Ca\(^{2+}\)-sensitive K\(^+\) currents. Taken together, these results suggest that neuropathic pain may be mediated, in part, by loss of I\(_{\text{Ca}}\) and the cellular processes dependent on Ca\(^{2+}\).

FEW conditions resist effective treatment like pain resulting from nerve injury. For patients with limb trauma or chronic diseases such as diabetes or tumor formation, the cost of untreated pain is loss of function or total incapacity. Animal models in which the peripheral nerve is incompletely injured result in heightened reaction to mechanical stimulation indicative of neuropathic pain1. Explaining the molecular and chemical bases of these changes will suggest means of better treatment.2

We have reported that chronic constriction injury3 of the sciatic nerve reduces calcium currents (I\(_{\text{Ca}}\)) in somata of primary afferent neurons in the dorsal root ganglia (DRG) proximal to the injury. These findings imply that decreased I\(_{\text{Ca}}\) may contribute to aberrant sensory processing, but it is not known whether I\(_{\text{Ca}}\) loss is solely a direct effect of axotomy or also an indirect effect of injury on adjacent, intact neurons. The spinal nerve ligation (SNL) model of neuropathic pain4 segregates the somata of axotomized L5 neurons from the adjacent L4 neurons that share the sciatic nerve, where intact L4 axons are exposed to inflammatory mediators induced by the degeneration of distal segments of L5 neurons.5–7 This model thus provides the opportunity to separately test the roles of neurons affected by these different pathogenic processes. Because the relative roles of axotomized and neighboring intact neurons are unresolved,8 we compared neurons from the ligated L5 DRG and the adjacent L4 DRG.9,10 We hypothesized that I\(_{\text{Ca}}\) loss is a generalized effect of painful sensory neuron injury and would be found after SNL as well as after chronic constriction injury. We further expected that the loss of current would be predominantly expressed in directly injured L5 neurons as compared with L4 neurons.

Voltage-activated Ca\(^{2+}\) channels contribute to shaping the action potential (AP) profile and neuronal excitability.11,12 To determine the biophysical role of decreased I\(_{\text{Ca}}\), we measured the effects of injury on both the AP parameters and the underlying I\(_{\text{Ca}}\) in the same neurons. The normal AP of sensory somata is only a few milliseconds long, so the response to conventional patch clamp test pulses lasting hundreds of milliseconds may not accurately depict I\(_{\text{Ca}}\) during normal neuronal activity. By exposing each cell to a voltage command in the shape of an AP waveform, we measured total charge transfer as a representation of net Ca\(^{2+}\) entry into the cell under natural physiologic conditions. Finally, complex interactions of various currents make prediction of net effects of the change in any one current difficult to predict. Specifically, inward Ca\(^{2+}\) current depolarizes the membrane, but the arrival of Ca\(^{2+}\) in the cytoplasm triggers the opening of Ca\(^{2+}\)-activated K\(^+\) channels that conduct an outward repolarizing current I\(_{\text{K(Ca)}}\). The balance of these two actions dictates the outcome of decrease I\(_{\text{Ca}}\) on neuronal function. We therefore determined the direct consequences of I\(_{\text{Ca}}\) loss on AP characteristics and overall membrane currents using selective Ca\(^{2+}\) channel antagonists.

Materials and Methods

Animal Preparation

Two hundred forty-five cells were derived from 98 adult male Sprague-Dawley rats weighing 125–150 g...
provided by a single vendor (Charles River Laboratories Inc., Wilmington, MA), after approval of the Medical College of Wisconsin Institutional Animal Care and Use Committee (Milwaukee, Wisconsin). An additional 67 cells obtained from 21 adult male Sprague-Dawley rats (Taconic Farms, Inc., Hudson, NY) were studied in a separate group. Rats received either ligation and section of the right L5 and L6 spinal nerves (SNL) approximately 5 mm distal to the DRG4 or control surgery with skin incision only. After fully recumbent recovery from halothane anesthesia, rats were returned to individual cages under climate- and light-controlled conditions for 10 days before behavioral testing as previously described. Rats were weighed and placed on a 0.25-in wire grid in clear plastic enclosures and allowed to rest for 30 min. Five applications of a 22-gauge spinal needle were made to the plantar skin of each paw and repeated 5 min later, using a force adequate to indent the skin but not to puncture it. These mechanical stimuli produced either a normal brief flinch, or a hyperalgesic-type response characterized by sustained (> 2 s) paw lifting, shaking, and licking. Only SNL rats with an ipsilateral response rate of at least 20% averaged over 3 test days and normal contralateral responses were used for study. The validity of this approach to behavioral testing is supported by the observation that control rats never exhibited characteristic hyperalgesic responses.

Cell Isolation and Solutions

After a postsurgical interval of 15.8 ± 1.4 days, the L4 and L5 ganglia were removed after decapitation during halothane anesthesia, placed into separate 35-mm Petri dishes containing Ca2+ and Mg2+-free iced Hanks’ Balanced Salt Solution, and minced with iris scissors. Using separate sterile, silicone-coated (Sigmacote; Sigma, St. Louis, MO) Pasteur pipettes, each ganglion was placed into 25-ml sterile tissue culture flask containing 0.0625% trypsin (Boehringer-Mannheim, Indianapolis, IN), 0.0125% DNase (Sigma), and 0.01% blendzyme 2 (Roche Molecular Biochemicals, Indianapolis, IN) in DMEM/F12 with glutaMAX (Invitrogen Corporation, Carlsbad, CA) for 1.5 h in a tissue shaking bath at 32°C and 70 rpm. Neurons were resuspended in adult neural basal media (1×: Invitrogen Corporation, Carlsbad, CA) containing 2% (vol:vol) B27 supplement (50×: Invitrogen Corporation), 0.5 mM glutamine, 0.02 mg/ml gentamicin, and 100 ng/ml nerve growth factor 78 (Alomone Labs, Ltd., Jerusalem, Israel). Dissociated L4 and L5 cells from each tube were plated onto separate poly-L-lysine (70–150 kd)—coated coverslips and placed in a 95:5 oxygen–carbon dioxide, water-jacketed incubator and allowed to plate for 2 h before study. All cells were studied within 8 h after plating.

Current clamp solutions duplicated natural cytosolic and extracellular conditions. A modified Tyrode solution was used externally in the bath during current clamp protocol, consisting of the following: 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM D-glucose, and 10 mM HEPES at a pH of 7.4 with NaOH and an osmolality of 300 mOsm. Internal pipette solution contained 120 mM KCl, 5 mM Na-ATP, 0.4 mM Na-GTP, 5 mM EGTA, 2.25 mM CaCl2, 5 mM MgCl2, and 20 mM HEPES at a pH of 7.4 with 2-ME and an osmolality of 296 mOsm. Because internal pipette solutions cannot be changed after seal formation, ICa was isolated by external blockade of other voltage-sensitive currents. The following solution provided adequate separation from other currents: 2 mM BaCl2, 1 mM 4-aminopyridine, 10 mM HEPES, 160 mM tetraethylammonium chloride, and 0.1 mM tetrodotoxin at a pH of 7.4 with tetraethylammonium hydroxide and an osmolality of 300 mOsm. Tetrodotoxin blocked tetrodotoxin-sensitive INa, and 4-aminopyridine, tetraethylammonium chloride, and Ba2+ blocked potassium currents. Because the change in external solutions results in a +4 mV shift in junction potential, no equivalent offset was added after switching from current clamp to voltage clamp. Cadmium (200 μM) was added at the end of some studies to verify the efficacy of ICa isolation. In a separate protocol examining AP parameters and whole cell currents before and after ICa blockade, Ca2+ channel antagonists were applied directly to the cell through a pressure regulated microperfusion system (ALA-BP8 system; ALA Scientific Instruments, Westbury, NY). Complete ablation of high voltage–activated Ca2+ channels was achieved with a cocktail of antagonists that contained nisoldipine (200 nM), SNX-111 (200 nM), agatoxin IVA (200 nM), and SNX-482 (200 nM) to block L-, N-, P/Q-, and R-type calcium channel subtypes, respectively. Tetrodotoxin was obtained from Alomone Labs. SNX-111 was the kind gift of Dr. Scott Bowersox, Ph.D. (Department of Pharmacology, Neurex Corporation, Menlo Park, CA), agatoxin IVA was a gift of Dr. Nicholas Saccomano, Ph.D. (Assistant Director, Medical Chemistry Research at Pfizer, Groton, CT), and SNX-482 was purchased from Peptides International (Louisville, KY).

Recording Protocols

All cells were recorded in current clamp before changing to voltage clamp. Cells with resting membrane potentials more depolarized than −45 mV were excluded from study. Efficacy of potassium and sodium channel blockade was confirmed by observation of a reversal of negative membrane potential while still in current clamp and a lack of brief, inward currents during voltage clamp recording. Terminal application of Cd2+ to block ICa showed no residual voltage-activated currents. Membrane capacitance was measured and access resistance compensated from 80 to 95%. Cells with greater than 10 MΩ access resistance were rejected. Most recordings were made on a List EP-7 amplifier (ALA Scientific Instruments) modified by the addition of a 150-kΩ resistor to the current injection circuit to increase maximum stim-
the beginning of the rapid upstroke, whereas AP duration at 50% of peak (AP<sub>50</sub>). Inflection on the repolarization phase of the AP was observed, and the number of APs during 1 s current injection was determined to characterize the neuron as either adapting or repetitive firing. Continuous current injection and positive feedback from patch clamp head stage amplification prolongs AP duration. Nevertheless, the relative effect of injury is still detectable. To avoid distortion from continuous current injection during measurement of the afterhyperpolarization (AHP), additional APs were evoked by a brief, 2-ms, 5-nA depolarizing pulse, and peak hyperpolarization (AHP<sub>ampl</sub>) and duration at 50% amplitude (AHP<sub>50</sub>) were recorded, as well as the time constant for resolution of the AHP (AHP<sub>r</sub>) by fitting the recovery phase to a single exponential (fig. 1).

**Voltage Clamp.** All cells were exposed to both a sustained square wave protocol and a standardized AP waveform voltage command protocol. The square wave protocol consisted of 200-ms commands from a holding potential of −90 to +50 mV in 10-mV increments with 5-s intervals between steps. Measured inward current was normalized by membrane capacitance, which results in a current density corrected for cell size. Peak inward current was determined, and linear leak was subtracted post hoc by fitting the first three steps before significant I<sub>Ca</sub> activation to a linear function. Maximum conductance (G<sub>max</sub>) was determined by fitting peak inward current to a Boltzmann function, as previously described. Some large cells produced currents in excess of the bandwidth of the List EP-7 amplifier. Therefore, maximal I<sub>Ca</sub> for large cells was calculated by linear fit of current-voltage peaks from the three traces before and at the reversal potential. G<sub>max</sub> determined by this procedure differed from that determined by Boltzmann fit by ±3% in 12 nonsaturating cells.

A standardized AP waveform command was used to reveal the effects of naturally rapid depolarization and repolarization on I<sub>Ca</sub> across all cells. The basic features of the standardized wave were an inflection on the repolarization phase and a prolonged AHP to mimic standard features of a nociceptor (fig. 1). A modified P8 wave was used to subtract capacitance; namely, the AP waveform was inverted and divided by 8 to acquire membrane capacitance with minimal channel activation. Each P8 wave was subsequently multiplied by −8 and subtracted from the current in response to an AP waveform voltage command. Peak inward current and total charge transfer (current integrated over time) were determined by cursor measurements after current traces crossed 0 mV at the beginning and end of a wave (fig. 1). Charge transfer (coulombs) was normalized to cell area by converting cell capacitance to the specific capacitance of plasma membranes (0.01 pF/μm<sup>2</sup>) to compare Ca<sup>2+</sup> flux between cells of different sizes. Because cells reliably produced a triangular current waveform (fig. 1), peak and area for large cells with saturating currents were calculated to 5 nA. An A/D converter (Axon 1200B; Axon Instruments, Union City, CA) attached to a personal computer running pClamp 8 (Axon Instruments) was used to record data. A second series of experiments on large and medium cells with I<sub>Ca</sub> of up to 72.5 nA were performed on an Axon 200B amplifier (Axon Instruments) with a different A/D converter (Axon 1320; Axon Instruments).

**Current Clamp.** Initial observation of resting membrane potential determined cell viability before evoked potentials. To determine AP characteristics, a 1-s step protocol was used with current sufficient (between 10 pA and 2 nA) to evoke an AP (fig. 1). AP voltage threshold (AP<sub>thresh</sub>) was defined as the level of the inflection at the beginning of the rapid upstroke, whereas AP duration was measured at 50% of peak (AP<sub>50</sub>). Inflection on the repolarization phase of the AP was observed, and the number of APs during 1 s current injection was determined to characterize the neuron as either adapting or repetitive firing. Continuous current injection and positive feedback from patch clamp head stage amplification prolongs AP duration. Nevertheless, the relative effect of injury is still detectable. To avoid distortion from continuous current injection during measurement of the afterhyperpolarization (AHP), additional APs were evoked by a brief, 2-ms, 5-nA depolarizing pulse, and peak hyperpolarization (AHP<sub>ampl</sub>) and duration at 50% amplitude (AHP<sub>50</sub>) were recorded, as well as the time constant for resolution of the AHP (AHP<sub>r</sub>) by fitting the recovery phase to a single exponential (fig. 1).

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**Table 1. Injury Effects on AP Parameters and I\_ca**

<table>
<thead>
<tr>
<th>Command</th>
<th>Injury Model</th>
<th>n</th>
<th>(\text{AP}_{50}), ms</th>
<th>(\text{AP}_{\text{thresh}}), mV</th>
<th>(\text{AHP}_{\text{amp}}), mV</th>
<th>(\text{AHP}_{\text{dur}}), ms</th>
<th>(\text{G}_{\text{max}})</th>
<th>pS/(\mu)F</th>
<th>(I_{\text{peak}})</th>
<th>pA/(\mu)F</th>
<th>Charge Transfer, (\text{fC}/\mu)m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>Control</td>
<td>20</td>
<td>4.4 ± 0.4</td>
<td>10.1 ± 2.0</td>
<td>15.8 ± 1.5</td>
<td>38.3 ± 4.8</td>
<td>55.7 ± 9.5</td>
<td>3.7 ± 0.3</td>
<td>-249.9 ± 19.8</td>
<td>4.66 ± 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNL L4</td>
<td>20</td>
<td>5.0 ± 0.5</td>
<td>-11.5 ± 2.2</td>
<td>-16.6 ± 1.2</td>
<td>34.3 ± 7.3</td>
<td>41.0 ± 10.6</td>
<td>3.1 ± 0.4</td>
<td>-220.3 ± 29.1</td>
<td>4.08 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>Control</td>
<td>19</td>
<td>14.8 ± 0.3*</td>
<td>14.7 ± 1.8*</td>
<td>14.9 ± 1.4</td>
<td>42.7 ± 7.2</td>
<td>53.2 ± 12.2</td>
<td>2.3 ± 0.2*</td>
<td>-143.1 ± 17.2*</td>
<td>2.76 ± 0.40*</td>
<td></td>
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<tr>
<td></td>
<td>SNL L4</td>
<td>14</td>
<td>4.6 ± 1.1</td>
<td>-22.4 ± 3.4</td>
<td>-9.1 ± 1.8*</td>
<td>45.4 ± 11.3</td>
<td>70.8 ± 15.9</td>
<td>2.6 ± 0.4</td>
<td>-114.1 ± 21.6*</td>
<td>2.52 ± 0.57*</td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>L5</td>
<td>24</td>
<td>6.4 ± 1.1*</td>
<td>-19.4 ± 1.7</td>
<td>-10.9 ± 0.8*</td>
<td>20.8 ± 2.9*</td>
<td>29.6 ± 4.9*</td>
<td>2.2 ± 0.3*</td>
<td>-113.6 ± 16.5*</td>
<td>2.54 ± 0.37*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNL L4</td>
<td>16</td>
<td>3.6 ± 1.4</td>
<td>16.7 ± 3.2</td>
<td>14.9 ± 1.2</td>
<td>90.5 ± 14.1</td>
<td>181.6 ± 23.6</td>
<td>3.9 ± 0.5</td>
<td>-192.2 ± 21.4</td>
<td>5.39 ± 16.4*</td>
<td></td>
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<tr>
<td></td>
<td>L4</td>
<td>13</td>
<td>2.3 ± 0.3</td>
<td>-20.8 ± 3.2</td>
<td>-15.4 ± 1.8</td>
<td>53.0 ± 14.8</td>
<td>94.8 ± 29.5*</td>
<td>2.4 ± 0.5*</td>
<td>-104.0 ± 30.4*</td>
<td>2.05 ± 0.76*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L5</td>
<td>12</td>
<td>3.1 ± 0.5</td>
<td>-17.7 ± 2.1</td>
<td>-10.5 ± 1.3*</td>
<td>57.7 ± 19.8*</td>
<td>115.5 ± 46.3*</td>
<td>2.3 ± 0.5*</td>
<td>-105.3 ± 26.1*</td>
<td>1.87 ± 0.51*</td>
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</table>

Injury prolonged action potential (AP) in small and medium neurons, whereas afterhyperpolarization (AHP) was decreased in medium and large cells in response to current clamp command protocols. Every group lost Ca\(^{2+}\) current (\(I_{\text{ca}}\)) in response to voltage clamp command protocols after injury. Neurons grouped by cell size and injury model were analyzed by comparison with “control” neurons from skin-sham operated rats. Means compared with analysis of variance and Bonferroni post hoc, except in large cell voltage clamp data, where nonparametric Kruskal-Wallis with Dunn post hoc comparisons were used to account for signal saturation. See Materials and Methods.

Statistical Analyses

Cells were categorized according to cell size and surgical preparation. As noted elsewhere, \(^{16,17}\) cell size correlates roughly to neuronal types, so we classified cells as small (C type, < 30 \(\mu\)m), medium (A\(\delta\) type, 30–40 \(\mu\)m), or large (A\(\alpha/A\(\beta\) type, > 40 \(\mu\)m). Neurons were further grouped according to surgical preparation (control or SNL) and DRG level (L4 or L5). Because there were no differences in responses from L4 and L5 ganglia from control rats, these cells were combined, resulting in a control group, an SNL L4 group, and an SNL L5 group. Recordings were evaluated post hoc using Clampfit 8 (Axon Instruments), and data were summarized in Excel (Microsoft Co., Redmond, WA) and analyzed with Statistica 6 (StatSoft, Tulsa, OK) for analysis of variance and paired \(t\) tests or GraphPad Prism 4 for Mac (Graph-Pad Software, San Diego, CA) for correlations. Values are expressed as mean ± SEM. For each dependent variable, the main effect of a group was tested with standard univariate analysis of variance, followed by planned Bonferroni post hoc comparisons between groups. Normal distribution was not assumed for large cells in voltage clamp mode (confirmed by Kolmogorov-Smirnov test for normality) because of occasional outliers with very large currents. For this size group, a nonparametric Kruskal-Wallis analysis was used with the Dunn test for post hoc comparisons. Significance was estimated at \(P \leq 0.05\) versus control. Drug effects were evaluated with paired \(t\) tests for dependent samples before and after drug administration. Pearson tests were calculated to determine the coefficient of correlation between \(G_{\text{max}}\) in voltage clamp and AP or AHP parameters in current clamp. Two-tailed \(P\) values and confidence levels were computed to determine whether covariance \((\chi^2)\) was different from zero.

Results

Current Clamp

Injury was associated with longer action potential duration (\(\text{AP}_{50}\)) in small and medium neurons from L5 ganglia compared with control cells, whereas medium and large cells from the same ganglion were characterized by AHPs with lower amplitudes and shorter durations after injury (table 1 and fig. 2). The \(\text{AP}_{\text{thresh}}\) was significantly reduced only in small cells from the L5 DRG of SNL rats compared with control neurons.

Inflection of the descending limb of the AP identifies nociceptors.\(^18\) Overall, inflected cells had a longer \(\text{AP}_{50}\) (6.4 ± 0.6 vs. 3.3 ± 0.5 ms, \(P < 0.05\)) and depolarized threshold (−15.4 ± 0.8 vs. −23.2 ± 1.2 mV, \(P < 0.05\)). Comparing neurons with and without inflection did not show any difference in the response of AP parameters to injury in any surgical group.

Cells from adjacent, noninjured L4 ganglia after SNL were statistically indistinguishable from control neurons in most parameters, except for isolated changes after injury in AHP amplitude in medium neurons and AHP duration in large neurons. Therefore, cells from adjacent,
nonligated ganglia did not show the consistent pattern of AP or AHP parameter changes demonstrated in cells from the L5 ganglia of injured rats.

Voltage Clamp

Voltage clamp studies were conducted in the same cells after fluid change. Efficacy of potassium and sodium channel blockade was confirmed by observation of elimination of the negative membrane potential while still in current clamp and lack of brief, inward currents during voltage clamp recording. In addition, terminal application of Cd²⁺/H₁₁₀₀₁ to block I_{Ca} showed no residual voltage-activated currents.

Spinal nerve ligation reduced Ca²⁺ conductance in L5 neurons across all size groups compared with neurons from control rats. Specifically, Gₘₐₓ was reduced across all three size groups from 36 to 37% in cells from L5 SNL ganglia compared with neurons from control rats. Reduced I_{Ca} was also reflected in responses to AP waveform commands. Specifically, peak current amplitude and the integral of Ca²⁺ flux over the course of the AP waveform (total charge transfer) fell between 37 and 60% in L5 ganglia from SNL rats compared with control. Less consistent decreases were found during voltage clamp examination of L4 neurons (table 1), which showed reduced Gₘₐₓ only in large neurons and altered AP waveform responses only in medium and large neurons.

Only L5 neurons showed decreased I_{Ca} after injury in the small neuron group, whereas there were changes in both L4 and L5 neurons in the medium and large groups. However, this part of the study was not specifically designed to compare L4 and L5 neurons. To directly assess the possibly distinct effects of injury on L4 and L5 neurons, we devised additional studies to optimally compare currents in these two groups. In these experiments, we examined currents in paired L4 and L5 medium and large neurons isolated from the same animal under identical conditions. This focused examination also eliminated inconsistencies that accrue due to data collection over a span of time (2 yr for the data set above), including genetic drift in rat breeding colonies and different batches of dissociation enzymes. Further, we used a different amplifier with expanded bandwidth to clamp whole cell recordings with up to 72.5 nA peak inward current. These experiments identified contrasting injury responses in medium and large L4 and L5 neurons. Specifically, I_{Ca} in L4 neurons from medium cells was unchanged, whereas L5 neurons displayed reduced I_{Ca} compared with L4 and control neurons (fig. 3). I_{Ca} in large L4 and L5 neurons did not differ from control.

Fig. 2. Exemplary traces showing effects of injury on action potential dimensions in control neurons and after ligation and axotomy of the fifth lumbar spinal nerve (SNL L5). (A) A typical small neuron displays prolonged action potential duration and a more depolarized resting membrane potential after injury. (B) A medium neuron exhibits prolonged action potential as well as decreased afterhyperpolarization amplitude and accelerated recovery of the afterhyperpolarization after spinal nerve ligation. (C) A large neuron develops decreased afterhyperpolarization amplitude and duration. (D) The stimulus in each case is a sustained current pulse of an amplitude just adequate for action potential generation. Scale bars apply to all traces.

Fig. 3. Voltage clamp data comparing paired sensory neurons from the L4 and L5 levels after spinal nerve ligation (SNL) and neurons from control animals, including large and medium groups. No differences were discerned in large cells (A). Medium cells demonstrated significant difference in maximum Ca²⁺ conductance (Gₘₐₓ) in response to 200-ms, 10-mV, stepwise, square wave pulses (B), and in peak amplitude (C) and total charge transfer (D) in response to a standardized action potential waveform. n = 7–13 neurons for each group. Peak current is normalized for cell capacitance; charge transfer is normalized for cell surface area. § P < 0.05 versus both L4 and control, by Kruskal-Wallis nonparametric analysis of variance with Dunn multiple comparison test for post hoc differences.
Table 2. Effect of Ca\(^{2+}\) Channel Antagonists

<p>|                | Voltage Clamp | Current Clamp |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>I(_{\text{peak}}) Inward, pA/pF</td>
<td>I(_{\text{peak}}) Outward, pA/pF</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>−239.5 ± 49.2</td>
<td>269.3 ± 38.0</td>
</tr>
<tr>
<td>Blockade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>−126.9 ± 26.6'</td>
<td>147.5 ± 27.5'</td>
</tr>
</tbody>
</table>

Ca\(^{2+}\) channel antagonists mimic injury effect. Blockade with specific high voltage-activated Ca\(^{2+}\) channels prolonged action potential (AP) duration and decreased afterhyperpolarization (AHP) amplitude in current clamp mode, whereas peak inward and outward currents were decreased in the same cell in voltage clamp mode.

\( ^* P \leq 0.01\) vs. control, paired t test.

AHP\(_{\text{ampl}}\) = AHP amplitude; AP\(_{\text{dur50}}\) = AP duration at 50%; I\(_{\text{peak}}\) Inward, Outward = peak inward and outward voltage clamped currents in response to AP waveform from same cell.

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Because reduced I\(_{\text{Ca}}\) may have diverging effects on the AP depending on whether the direct inward Ca\(^{2+}\) current or the induced outward I\(_{\text{K(Ca)}}\) dominates, we correlated G\(_{\text{max}}\) against AP parameters measured during current clamp in the same cells to determine the role of I\(_{\text{Ca}}\) in AP\(_{\text{dur50}}\) correlated negatively with G\(_{\text{max}}\) in small cells from L5 of injured rats (fig. 4A) but positively in large control and SNL L5 neurons (fig. 4B), indicating opposite contributions of I\(_{\text{Ca}}\) to AP duration in these two neuronal groups. While G\(_{\text{max}}\) did correlate positively with AP\(_{\text{dur50}}\) in large L5 neurons after SNL, this correlation was also seen in large cells from control ganglia without difference in means as determined by analysis of variance (table 1).

Small control neurons showed minimal dependence of AP repolarization on Ca\(^{2+}\)-dependent processes (fig. 4A). G\(_{\text{max}}\) correlated negatively with AHP\(_{\text{ampl}}\) in large cells from L5 ganglia of injured rats (fig. 4C). G\(_{\text{max}}\) did not correlate with any parameters in L4 and medium-sized neurons.

I\(_{\text{Ca}}\) Blockade

To identify the biophysical consequences of reduced I\(_{\text{Ca}}\), we duplicated the loss of I\(_{\text{Ca}}\) seen after injury by application of Ca\(^{2+}\) channel blockers and measured the effect of blockade on AP parameters and whole cell currents in the same cell (table 2). Medium or small neurons (n = 9) from nonoperated rats were studied in normal Tyrode solution before and after complete I\(_{\text{Ca}}\) ablation with a cocktail of high voltage-activated Ca\(^{2+}\) channel antagonists containing nisoldipine (200 nm), SNX-111 (200 nm), agatoxin IVA (200 nm), and SNX-482 (200 nm) to block L-, N-, P/Q-, and R-type calcium channel subtypes, respectively. An AP was elicited with a brief (0.5-ms) pulse ranging from 1 to 3 nA. This same AP was used as a voltage command to elicit whole cell currents in the same cell. After capacity transients, an inward current representing calcium and sodium was followed by an outward current representing potassium (fig. 5). Calcium channel blockade significantly prolonged average AP\(_{\text{dur50}}\) and decreased AHP\(_{\text{ampl}}\) during current clamp recordings (fig. 5A), whereas underlying inward and outward currents were significantly reduced. It is noteworthy to observe the time course of ionic changes (fig. 5B). Reduced inward I\(_{\text{Ca}}\) correlated with the delayed pace of AP depolarization, whereas outward current, presumably I\(_{\text{K(Ca)}}\), was reduced during the repolarization phase of the AP and the onset of AHP. This provides evidence of a critical role of I\(_{\text{Ca}}\) in production of outward currents during AP repolarization in sensory neurons.
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which neurons injured by complete axonal transection
behavior, so we chose a model of neuropathic pain in
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larization (deactivation) of various currents critically
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Discussion
In this study, we have observed decreased I\(_{Ca}\) in the
somata of sensory neurons after peripheral nerve injury
by SNL. In combination with our previous findings after
more peripheral sciatic injury by chronic constriction,\(^{19}\)
this indicates that I\(_{Ca}\) is a general feature of nerve injury.
The chronic constriction injury model of neuropathic
pain is limited by the inability to distinguish the contrib-
ution of axotomy versus inflammatory mechanisms,
because somata of axotomized neurons cannot be
readily distinguished from those with intact axons.
In-complete injury is a prerequisite for provoked pain be-
behavior, so we chose a model of neuropathic pain in
which neurons injured by complete axonal transection
might be compared with anatomically segregated neigh-
bring neurons. We have found that reduced I\(_{Ca}\) is pre-
dominantly a consequence of axotomy (L5 after SNL)
and is most evident in small and medium neurons.
Various voltage-activated currents, especially N and T
types, inactivate during sustained depolarization,\(^{20,21}\) so
dynamic aspects of voltage commands may substantially
affect patterns of recorded I\(_{Ca}\). Studies of ionic currents
underlying neuronal membrane function typically rely
on prolonged command pulses to detect voltage sensi-
tivity and kinetic properties of channel gating. However,
natural neuronal activity leads to opening of voltage-
gated channels in response to only brief membrane de-
polarization events, under which conditions the specific
activation, inactivation, and channel closure after repo-
larization (deactivation) of various currents critically
controls Ca\(^{2+}\) flux. We therefore used a voltage com-
mand in the form of an AP waveform to determine whether
an injury would produce a loss of I\(_{Ca}\) as has been
observed with prolonged step protocols. We find that in-
jury reduces I\(_{Ca}\) in response to an AP waveform to an
even greater extent than I\(_{Ca}\) measured in response to
200-ms step pulses, which extends observations from
traditional sustained square wave protocols and confirms
that loss of I\(_{Ca}\) represents a true biophysical change
associated with neuropathic pain.

We categorized neurons by size, whereas other studies
have used AP characteristics, expression of membrane
markers, or sensitivity to neuropeptides and algogenic
agents. Clearly, sensory neurons show a high degree of
heterogeneity, with some investigators describing seven
subtypes in small cells alone.\(^{22}\) However, nerve injury
causes shifts in all these markers,\(^{23,24}\) making their use
problematic for categorizing neurons. A further limita-
tion is that the exact electrophysiologic changes induced
by injury in AP parameters are technique dependent.
Specifically, intracellular recordings from nondissociated
sensory neurons by us and others\(^{21,25}\) contrast with the
current patch recordings from dissociated neurons in
showing prolonged AP duration after injury in large but
not small neurons. To address these limitations, the cur-
rent study examined Ca\(^{2+}\) membrane currents together
with AP characteristics in the same neuron, thus directly
highlighting the role of I\(_{Ca}\).

The functional implications of I\(_{Ca}\) loss are not fully
understood. Previous reports have shown that Ca\(^{2+}\) con-
tributes a large inward current during the repolarization
phase of the AP, leading to a prolongation or inflection
of the descending limb, whereas removing Ca\(^{2+}\) short-
ens AP duration.\(^{26–28}\) Accordingly, we have observed a
direct relation between AP duration and I\(_{Ca}\) in large
neurons (fig. 3B).

Among small and medium neurons, however, dimin-
ished I\(_{Ca}\) prolongs rather than shortens the AP (table 1
and fig. 4). Blockade with specific Ca\(^{2+}\) channel antag-
ons indicates that AP prolongation in these neurons is
controlled by a shift in the balance of currents such that
the loss of inward I\(_{Ca}\) is overwhelmed by a much greater
loss of outward I\(_{K(Ca)}\) (fig. 5). This relation by which
decreased I\(_{Ca}\) leads to prolonged AP duration is mirrored
in the findings of others who have shown that a net
outward current can result from Ca\(^{2+}\) entry in neuronal
cell types with a predominant expression of large con-
ductance voltage- and Ca\(^{2+}\)-gated K\(^+\) channels.\(^{29,30}\)
Taken together, these results demonstrate the fine bal-
cence between ionic conductances and firing patterns
which are altered in different ways by trauma. AP pro-
longation in small and medium neurons after injury may
give rise to increased neurotransmitter release at synap-
tic junctions in the dorsal horn and thereby contribute to
hyperalgesia.\(^{31}\)

We have also shown that I\(_{Ca}\) loss is associated with
AHP shortening in medium and large neurons after in-
jury. Because currents through Ca\(^{2+}\)-activated K\(^+\) chan-
nels contribute substantially to the AHP,\(^{32,33}\) our finding of
shortened AHP provides further evidence of decreased
outward K⁺ current secondary to ICa loss in these cell groups, although injury and inflammation could also have direct effects on these K⁺ channels. Blocking Ca²⁺ currents with specific toxins similarly caused a reduction of AHP amplitude and duration through diminished activation of Ca²⁺-dependent outward current. AHP loss primes the neuron for increased repetitive firing, as we have demonstrated in excised ganglia after injury. Therefore, the deficiency of ICa we have observed in sensory neurons after injury may substantially contribute to increased excitability and production of neuropathic pain. Further demonstration of this mechanism may be the antinociception provided by intrathecal Ca²⁺ injection in mice that is eliminated by blockers of calcium-activated potassium currents (IK(Ca)) and the burning pain in humans after delivery of a solution containing the ICa channel blocker glibenclamide. Even in the absence of natural stimulation, axotomized neurons are particularly subject to chemical activation by catecholamines, proinflammatory cytokines, bradykinin and neurotrophins, and mechanical stimulation. Cross-excitation from adjacent intact neurons, such as those in the dorsal primary ramus of the segmental spinal nerve that remains intact after SNL, also excites the axotomized somata. Our findings indicating membrane hyperexcitability may amplify this induced L5 traffic, leading to hyperalgesic sensory events when naturally triggered L4 activity arrives in the hyperexcitable dorsal horn.

Fig. 6. Schematic diagram of reduced Ca²⁺ current (ICa) contribution to neuropathic pain. Injured nerve tissue distal to the spinal nerve ligation (SNL) undergoes Wallerian degeneration (dotted line), while neurons from the L5 dorsal root ganglion (DRG) are activated by movement, catecholamines, other algogenic agents and cross-excitation from adjacent intact neurons. Reduced ICa in Aβ/Ad and Aδ fibers shortens afterhyperpolarizations (AHPs), which contributes to burst firing. Loss of ICa impairs natural signal filtering at the T-branch, where the unmyelinated axon splits into spinal nerve and dorsal root branches. Reduced ICa also prolongs action potential (AP) duration, which may increase excitatory synaptic transmission in the dorsal horn (DH). Sparred nerves transmit activity evoked by stimulation in the receptive field, and these signals encounter DH neurons that are sensitized by L5 input. L4, L5 = neurons in the fourth and fifth lumbar ganglia.

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