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Painful Nerve Injury Decreases Resting Cytosolic Calcium Concentrations in Sensory Neurons of Rats

Andreas Fuchs, M.D., Philipp Lirk, M.D.,† Cheryl Stucky, Ph.D.,‡ Stephen E. Abram, M.D.,§ Quinn H. Hogan, M.D.§*

Background: **Neuropathic pain is difficult to treat and poorly understood at the cellular level. Although cytoplasmic calcium ($[Ca^{2+}]_c$) critically regulates neuronal function, the effects of peripheral nerve injury on resting sensory neuronal $[Ca^{2+}]_c$ are unknown.**

Methods: **Resting $[Ca^{2+}]_c$ was determined by microfluorometry in Fura-2 AM–loaded neurons dissociated from dorsal root ganglia of animals with hyperalgesia to mechanical stimulation after spinal nerve ligation and section (SNL) at the fifth and sixth lumbar (L5 and L6) levels and from animals after skin incision alone (control group). Axotomized neurons from the L5 dorsal root ganglia were examined separately from adjacent L4 neurons that share the sciatic nerve with degenerating L5 fibers.**

Results: **After SNL, large (34 μm or larger) neurons from the L4 ganglion showed a 29% decrease in resting $[Ca^{2+}]_c$, whereas those from the L5 ganglion showed a 54% decrease. Small neurons only showed an effect of injury in the axotomized L5 neurons, in which resting $[Ca^{2+}]_c$ decreased by 30%. A decrease in resting $[Ca^{2+}]_c$ was not seen in neurons isolated from rats in which hyperalgesia did not develop after SNL. In separate experiments, SNL reduced resting $[Ca^{2+}]_c$ in capsaicin-insensitive neurons of the L5 ganglion by 60%, but there was no change in neurons from L4. Resting $[Ca^{2+}]_c$ of capsaicin-sensitive neurons was not affected by injury in either ganglion. SNL injury decreased the proportion of neurons sensitive to capsaicin in the L5 group but increased the proportion in the L4 group.**

Conclusions: **Painful SNL nerve injury depresses resting $[Ca^{2+}]_c$ in sensory neurons. This is most marked in axotomized neurons, especially the large and capsaicin-insensitive neurons presumed to transmit nonnociceptive sensory information.**

PAIN due to nerve injury may accompany diverse diseases, such as diabetes, herpes zoster, herniated nucleus pulposus, or direct nerve trauma.[1–3] Patients may report either spontaneous pain, discomfort on innocuous stimulation (allodynia), or exaggerated pain on nociceptive stimulation (hyperalgesia).[3,4] Neuropathic pain may continue for years after the original injury has healed, and currently available treatment is successful in only a minority of patients.[5]

Widely distributed pathogenic processes contribute to neuropathic pain, including dysfunction at the site of injury and in the spinal cord and brain. In addition, important alterations contributing to increased excitability have been noted in the somata of sensory neurons proximal to the site of nerve trauma.[6–8] Substantial membrane modifications have been identified in dorsal root ganglion (DRG) neurons in various models of neuropathic pain.[9] Current through potassium channels is decreased,[10] expression of various sodium channel isoforms is altered,[11] and influx through high-voltage–activated[12] and low-voltage–activated calcium channels[13] is diminished. Calcium is particularly important because of its double role in neurons. Not only does the inward calcium current support action potential depolarization, but also, once in the cell, calcium serves as a key second messenger directing neuronal function and is closely regulated by a complex system of buffers, pumps, and release mechanisms.[14] Although cytoplasmic calcium ($[Ca^{2+}]_c$) critically regulates neurotransmitter release, intracellular signaling cascades, membrane channel function, cell differentiation, and programmed cell death, there has been no investigation of effects of peripheral nerve injury on sensory neuronal $[Ca^{2+}]_c$.

The goal of this investigation was to identify effects of injury on resting $[Ca^{2+}]_c$ as a starting point in understanding the role of disturbed intracellular calcium signaling in neuropathic pain. We used digital microfluorometry to measure $[Ca^{2+}]_c$ in neurons dissociated from DRGs of control animals and animals with hyperalgesia from peripheral nerve trauma. Because there is uncertainty about the relative contributions of injured and adjacent neurons after nerve injury,[9] we determined resting $[Ca^{2+}]_c$ in neurons from the fourth (L4) and fifth lumbar (L5) DRGs after ligation and section of the fifth lumbar spinal nerve (SNL model). The L5 ganglion contains axotomized neurons, whereas the L4 DRG contains those that are intact but project axons that mingle in the sciatic nerve with degenerating fiber segments of L5 neurons (fig. 1), where they are exposed to an inflammatory milieu. DRG neurons are diverse, with cells responsive to low-threshold stimulation and others that are selectively responsive to high-intensity stimulation. We therefore categorized neurons by diameter because large somata size is an approximate indicator of nonnociceptive modality.[15,16] In separate experiments, we categorized neurons by sensitivity to capsaicin,[17] which is a hallmark of nociceptive modality.

Materials and Methods

All procedures were approved by the Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin.

* Research Fellow, Department of Anesthesiology, Medical College of Wisconsin. Staff Anesthesiologist, Department of Anesthesiology and Intensive Care Medicine, Medical University of Graz, Graz, Austria. † Research Fellow, Department of Anesthesiology, Medical College of Wisconsin. Resident, Department of Anesthesiology and Critical Care Medicine, Medical University of Innsbruck, Innsbruck, Austria. § Professor, Department of Anesthesiology, ‡ Assistant Professor, Department of Cell Biology, Medical College of Wisconsin.

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Address reprint requests to Dr. Hogan: Department of Anesthesiology, Medical College of Wisconsin, Room M4280, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. Address electronic mail to: qhogan@mcw.edu.

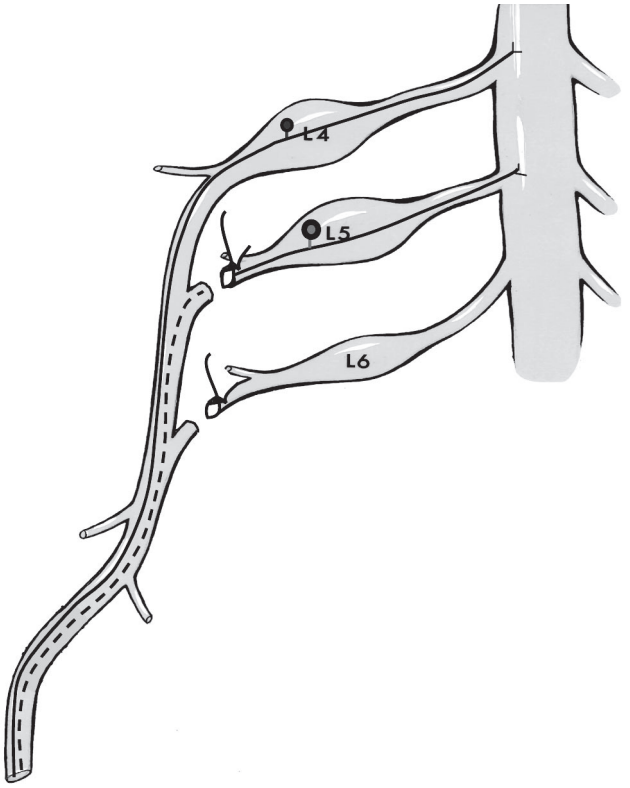

Fig. 1. Spinal nerve ligation model[18] of partial peripheral nerve injury, in which the spinal nerves (ventral primary ramus) at lumbar levels 5 and 6 are ligated and transected. The proximal site of this partial sciatic injury segregates axotomized L5 sensory neurons from intact neurons of the neighboring L4 ganglion, which share the sciatic nerve with the degenerating distal fragments of the L5 neurons (*dotted line*).

Injury Model

Male adult Sprague-Dawley rats (n = 42; Charles River Laboratories Inc., Wilmington, MA) weighing 160–180 g were randomly assigned to an SNL group or control group. SNL (n = 24 rats) was performed similar to the originally reported technique.[18] During anesthesia with halothane (2–3%) in oxygen, the right lumbar paravertebral region was exposed. After subperiosteal removal of the sixth lumbar transverse process, both the right fifth and the sixth lumbar spinal nerves were tightly ligated with 6-0 silk suture and transected distal to the ligature (fig. 1). No muscle was removed, the intertransverse fascia were incised only at the site of the two ligations, and articular processes were not removed. The lumbar fascia was closed by 4-0 resorbable polyglactin suture, and the skin was closed with three staples. In control rats (n = 18), only lumbar skin incision and closure was performed. After surgery, the rats were returned to the colony, where they were kept in individual cages under normal housing conditions.

Sensory Testing

Identification of hyperalgesia was conducted as previously described.[19] At least 1 day after arrival at the animal care facility, rats were brought to the testing area for 4 h of familiarization with handling and the environment. Hind paws were stimulated in random order with a 22-gauge spinal needle applied with pressure adequate to indent but not penetrate the plantar skin 2 days before surgery and on the 10th, 12th, and 14th postoperative days. Control rats showed only a brief withdrawal. SNL animals that displayed a hyperalgesia-type response with sustained lifting, licking, chewing, or shaking of the paw were considered to express a phenotype of neuropathic pain, whereas others without hyperalgesic responses were considered to lack neuropathic pain.

Cell Isolation and Plating

The L4 and L5 DRG were removed from control rats as well as both hyperalgesic and nonhyperalgesic SNL rats (studied separately) after halothane anesthesia and decapitation. The operative field was perfused with cold, oxygenated, calcium and magnesium chloride free Hanks Balanced Salt Solution. Minced ganglia were enzymatically dissociated in a solution containing 0.018% liberase blendzyme 2 (Roche Diagnostics Corp., Indianapolis, IN), 0.05% trypsin (Sigma, St. Louis, MO), and 0.01% deoxyribonuclease 1 (150,000 U; Sigma, St. Louis, MO) in 4.5 ml Dulbecco's modified Eagle's medium F12 (Gibco, Carlsbad, CA) for 90 min in a shaker bath at 32°C. Cells were harvested by centrifugation and resuspended in a culture medium consisting of 0.5 mM glutamine, 0.02 mg/ml gentamicin, 100 ng/ml nerve growth factor 7S (Alomone Labs, Jerusalem, Israel), 2% (vol/vol) B-27 supplement (Life Technologies, Rockville, MD), and 98% (vol/vol) neurobasal medium A 1X (Life Technologies) for plating onto poly-L-lysine–coated 12-mm glass coverslips (Deutsche Spiegelglas; Carolina Biologic Supply, Burlington, NC), plating from two to four slips per ganglion. Cells were incubated for 2–3 h in humidified incubator at 37°C with 95% air and 5% CO_2 before dye loading and were studied within 5 h of dissociation.

Calcium Microfluorometry

Cells were loaded with the ratiometric calcium indicator Fura-2 AM (2.5 μM in 0.1% Pluronic F-127; Molecular Probes, Eugene, OR) for 45 min at room temperature and then washed three times with a Tyrode's solution consisting of 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. Cells were left in a dark environment for 30 min for dye deesterification. Coverslips were mounted in a 500-μl recording chamber superfused with room-temperature (22°C) Tyrode's solution at a gravity-driven flow rate of 2 ml/min and imaged at 400× magnification using an inverted microscope (Nikon Diaphot 200; Tokyo, Japan) and cooled charge-coupled device camera (Cool Snap fx-Photometrics; Tucson, AZ). Cell diameter was determined by calibrated video image. On bright-field examination, neurons were excluded from measurement if

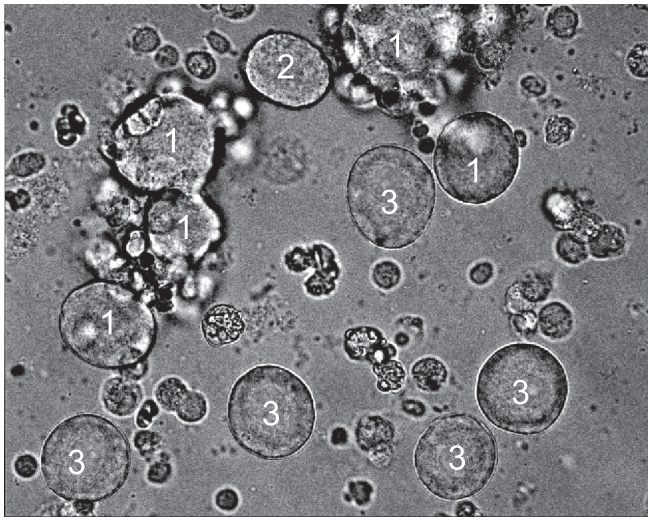

Fig. 2. Exclusion criteria by visual examination. Neurons were not used for data collection if there were overlying satellite cells (labeled *1*) or if the cell surface appeared wrinkled and collapsed (labeled *2*). All other neurons (labeled *3*) were included. Most fields showed a predominance of usable neurons. The full width of the image is 180 μm.

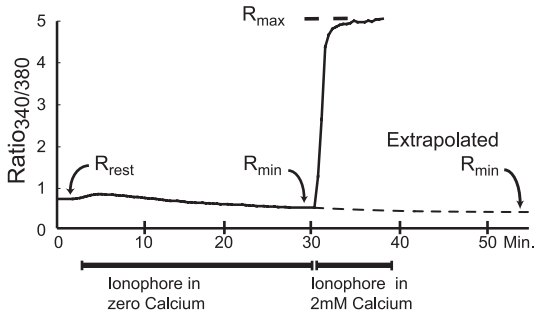

Fig. 3. Method of determination of calibration parameters for ratiometric microfluorometric cytosolic calcium concentration determination. The ratio (R) of emitted fluorescence intensity during 340-nm excitation divided by intensity during 380-nm excitation was measured at rest (R_{rest}), during ionophore and zero bath calcium, to determine the minimum fluorescence ratio (R_{min}) and during ionophore with 2 mm bath calcium to saturate the Fura-2 fluorophore and produce the maximum fluorescence ratio (R_{mzx}). Because R continues to decrease during R_{min} determination, an extrapolated value (Extrapolated R_{min}) was also determined by fitting an exponential decay curve, which in this case fit the data points with an r^2 value of 0.995.

they showed evidence of lysis or crenulation of their surface, because these cells showed unstable recordings, and also if they had overlying glial satellite cells (fig. 2). Each neuron was specified as a region of interest in the digital image (MetaFluor; Universal Imaging Corporation, Downington, PA) for separate R measurement, and an additional background area was recorded in each field for on-line subtraction of background fluorescence. Only one field was studied per slip. Autofluorescence of unloaded cells had a signal strength of less than 5% of the fluorescence of loaded cells. Emitted Fura-2 fluorescence was recorded at 510 ± 20 nm wavelength during alternating 340- and 380-nm excitation (DG-4; Sutter, Novato, CA). The frame capture period was 200 ms at intervals of 10 s. The ratio (R) of fluorescence excited by 340 nm divided by fluorescence excited by 380 nm was determined on a pixel-by-pixel basis, and $[Ca^{2+}]_c$ was calculated as

$$[Ca^{2+}]_c = K_d\beta(R - R_{min})/(R_{max} - R),$$

in which K_d is the dissociation constant for Fura-2 (specified as 224 nM),[20] R_{min} is the 340/380 fluorescence ratio in the presence of no calcium, R_{max} is the ratio in the presence of saturating concentrations of calcium, and β is the ratio of fluorescence during 380-nm excitation at zero and saturating calcium concentrations.

Resting R (R_{rest}) was measured by averaging 10 stable sequential determinations. In this study, R_{min}, R_{max}, and β were determined by *in situ* calibration for each cell, rather than applying averaged values as is typically done. Bath application of a calcium-selective ionophore (fig. 3), either ionomycin (5 μM) or 4-bromo A-23187 (5 μM; Molecular Probes), permitted equilibration of $[Ca^{2+}]_c$ with bath calcium concentration during superfusion first with Tyrode's solution with 10 mM EGTA and no added calcium to determine R_{min} and then with normal Tyrode's external solution to determine R_{max}.[21,22]

Drug Delivery

Drugs were delivered by bath change, except application of capsaicin, which was directed to the field through a microperfusion system 125 μm upstream from the imaged field. Using dye delivered through this system, onset time was measured as less than 200 ms, and there was no dilution of microperfusion solution despite bath flow.

Analysis and Statistics

Neurons were divided into two groups with diameters larger or smaller than 34 μm. This division is well established in similar studies of $[Ca^{2+}]_c$ in dissociated DRG neurons.[23,24] Although the size distribution of sensory neurons is a continuum (fig. 4),[25] grouping large and small neurons partially segregates the overlapping nociceptive and nonnociceptive neuron categories.[26–28] The

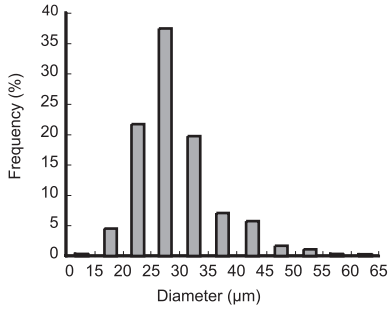

Fig. 4. Size histogram for recorded neurons. Total n = 1,057 cells.

chosen diameter for separating the large and small populations is derived from histologic studies.[29,30]

In separate experiments, neurons were categorized as sensitive to capsaicin if R increased by 50% over R_{rest}. Results are presented as mean ± SEM, and *n* refers to the number of cells tested unless otherwise stated. For $[Ca^{2+}]_c$ measurements, main effects were determined by two-way analysis of variance, and *post hoc* assessment of within-group comparisons was performed conservatively using the Bonferroni test (Statistica 6.0; StatSoft, Tulsa, OK). Differences between two groups were considered to be significant when *P* was less than 0.05. Significance of injury effects on frequency of capsaicin response was tested by nonparametric cross-tabulation. Because there is no established test for *post hoc* comparisons within contingency tables, values for Fisher exact test are reported. Concentration–response analysis was performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

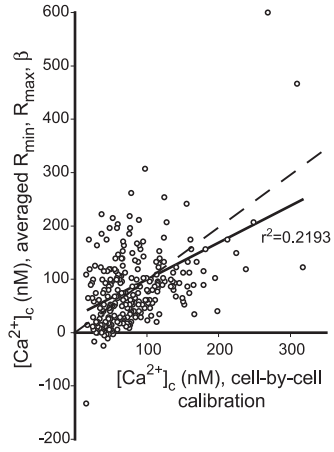

Fig. 5. Scatterplot comparing cytosolic calcium concentration ($[Ca^{2+}]_c$) calculated using either averaged calibration parameters R_{min}, R_{max}, and β (*ordinate*) or calibration parameters determined for each cell (*abscissa*). There is a poor correlation (*solid line*) between values (r^2 = 0.219), which is nonetheless significant (P < 0.001). The line of identity is shown in *dashes*.

Results

Behavioral Responses

Of 40 rats subjected to SNL, 24 in which hyperalgesia developed were considered successful neuropathic models, and DRG were removed 19.1 ± 0.5 days after injury. In these rats, hyperalgesia-type responses occurred in 41.4 ± 4.8% of needle applications to the right foot. Post mortem examination confirmed accurate placement of ligatures and section for all SNL animals. Hyperalgesic behavior did not develop in control rats (n = 17; 0 ± 0% hyperalgesic response rate; $P < 0.001$ *vs.* SNL), and DRG were removed 18.9 ± 1.6 days after skin incision surgery. Four rats in which hyperalgesia did not develop (1.7 ± 1.7% hyperalgesic response rate; $P < 0.01$ *vs.* SNL) were also studied at 19.5 ± 0.9 days after injury.

Comparison of Calibration Techniques

Stable R_{rest} recordings were achieved after 5–10 min of imaging for neurons dissociated from 65 DRGs (24 L4 and 24 L5 after SNL; 17 L4 or L5 control), including control cells (n = 343), SNL L4 cells (n = 252), and SNL L5 cells (n = 225). To determine whether extreme values of R_{min}, R_{max}, or β should be used as exclusion criteria in addition to visual features, we plotted $[Ca^{2+}]_c$ across the range of these parameters (data not shown). Because there were no outliers of $[Ca^{2+}]_c$ with the highest or lowest values of R_{min}, R_{max}, or β, we did not exclude any cells on this basis.

We noted that R continued to decrease during application of ionophore/low bath calcium for as long as 25 min (fig. 3). The descending trace of R during this interval fit an exponential decay curve (r^2 = 0.98 ± 0.06), so that an extrapolated R_{min} could be determined (Origin 7.0; Origin Lab Corp., Northampton, MA), which was lower than recorded R_{min} by an average of 0.10 ± 0.01 and resulted in an average increase of $[Ca^{2+}]_c$ by 26.4 ± 1.6 nM. We compared calibration using the ionophores 4-bromo A-23187 (n = 165) or ionomycin (n = 264). Because $[Ca^{2+}]_c$ decreased more slowly during R_{min} determination and cells more frequently detached from the coverslip during R_{max} determination using 4-bromo A-23187, further studies used only ionomycin as an ionophore during calibration. This choice is also supported by findings indicating that ionomycin has a higher selectivity for calcium and is more effective than 4-bromo A-23187 in binding and transporting calcium.[21] $[Ca^{2+}]_c$ values determined using 4-bromo A-23187 (n = 70) were higher in control animals than those (n = 122) determined using ionomycin, but the effects of injury on $[Ca^{2+}]_c$ were comparable (n = 164; data not shown). For ionomycin, average values of extrapolated R_{min}, measured R_{max}, and β were 0.53 ± 0.01, 5.19 ± 0.10, and 5.81 ± 0.13, respectively. Comparison of $[Ca^{2+}]_c$ calculated with either cell-by-cell calibration or mean values for R_{min}, R_{max}, and β (r^2 = 0.22; fig. 5) demonstrates the importance of determination of parameters for each cell. The absolute value of the difference between these two determinations is 58.7 ± 2.4 nM (overall for all cells), or 92 ± 6% of the fully calibrated $[Ca^{2+}]_c$.

Resting $[Ca^{2+}]_c$ Decreases after Injury in Large and Small DRG Neurons

Two-way analysis of variance showed significant main effects for both injury and neuronal size on $[Ca^{2+}]_c$, as well as a significant interaction of injury and size. Both large- and small-neuron groups showed significantly lower resting $[Ca^{2+}]_c$ in axotomized L5 neurons after SNL compared with control (fig. 6), but large neurons

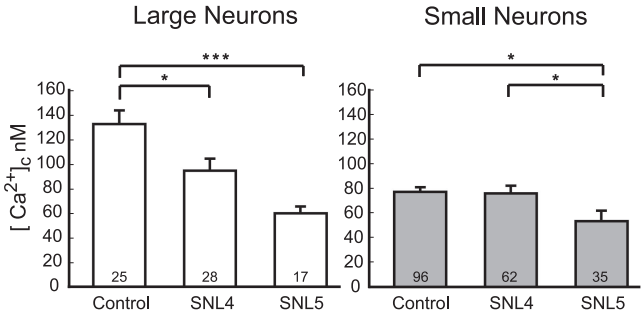

Fig. 6. Injury decreases resting cytosolic calcium concentration ($[Ca^{2+}]_c$) in large and small sensory neurons. Control neurons were compared to neurons from the L4 dorsal root ganglion (SNL4) and L5 ganglion (SNL5) after L5 and L6 spinal nerve ligation. Number of neurons for each group is shown in the *bars*. * $P < 0.05$, *** $P < 0.001$.

showed a proportionately greater decrease to 54% of control. Large neurons also showed a decrease of $[Ca^{2+}]_c$ in SNL L4 neurons to 29% of control.

Resting $[Ca^{2+}]_c$ Does Not Decrease after Injury in Rats in Which Hyperalgesia Does Not Develop

To probe whether there is a relation between the decreased $[Ca^{2+}]_c$ and altered sensory behavior after injury, we examined a small number of animals without behavior change. Unlike neurons from hyperalgesic rats, large neurons removed from these four rats did not show a decrease in resting $[Ca^{2+}]_c$ (182 ± 25 nM, n = 13 for L4; 146 ± 35 nM, n = 5 for L5) compared with neurons from control rats (133 ± 11 nM, n = 25). This was also true for small neurons from such rats, which showed significantly higher resting $[Ca^{2+}]_c$ in injured neurons (160 ± 15 nM, n = 22, $P < 0.001$ for L4; 129 ± 10 nM, n = 31, $P < 0.001$ for L5) compared with control (78 ± 4 nM, n = 96).

$[Ca^{2+}]_c$ Response to Capsaicin Increases in L4 Neurons but Decreases in L5 Neurons after SNL

Neurons with nociceptive and nonnociceptive modalities have broadly overlapping size distributions.[15,16] Because previous studies have established capsaicin sensitivity as a hallmark of nociceptors,[17] we categorized additional neurons according to their response to capsaicin, using a 50% increase of R to indicate sensitivity. A concentration-dependent response to capsaicin was evident between1 nM and 10 mM in uninjured neurons for both frequency of response at each dose and the response amplitude (fig. 7). A concentration of 100 nM resulted in an intermediate response rate comparable to the expected frequency of nociceptors among dissociated neurons and was used for further testing. For all control neurons, 63 of 130 (48%) responded to 100 nM capsaicin, with a smaller proportion in large neurons (5 of 20, 25%) than in small neurons (58 of 110, 53%; $P < 0.05$). SNL decreased the overall frequency of response to capsaicin in L5 neurons (7 of 57, 12%) but increased

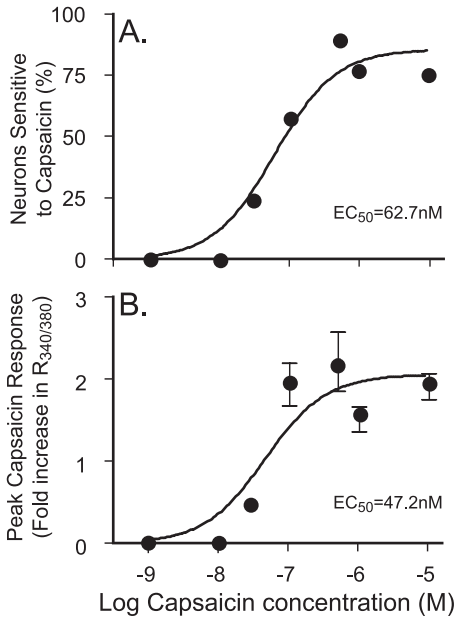

Fig. 7. Dose–response curves for increase of cytosolic calcium ($[Ca^{2+}]_i$) during capsaicin administration. (*A*) Frequency of at least 50% increase of fluorescence ratio ($R_{340/380}$) at each capsaicin concentration. (*B*) Magnitude of peak increase in $R_{340/380}$ at each capsaicin concentration. Line is fit by sigmoid dose–response curve (Prism 4.0; GraphPad Software Inc.).

the incidence in L4 neurons (51 of 77, 66%) compared with both control and SNL L5 (fig. 8). This influence of injury on capsaicin sensitivity was evident among both large and small neurons (data not shown).

Injury Decreases Resting $[Ca^{2+}]_c$ in Capsaicin-insensitive DRG Neurons

Two-way analysis of variance showed significant main effects for both injury and capsaicin sensitivity on resting $[Ca^{2+}]_c$. Separate analysis of insensitive neurons (fig. 9) showed that resting $[Ca^{2+}]_c$ for SNL L5 neurons was 60% lower than in control neurons and was also lower than in SNL L4 neurons. For capsaicin-sensitive neurons (fig. 9),

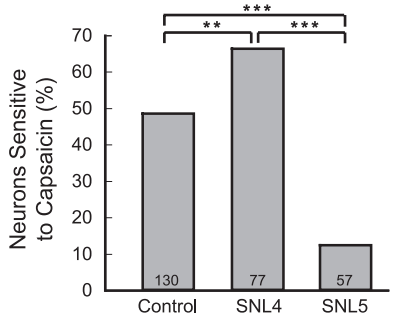

Fig. 8. Effect of injury on proportion of neurons that respond to capsaicin with an increase of cytosolic calcium. The threshold defining a sensitive neuron was a 50% increase of fluorescence ratio during 100 nM capsaicin administration. Control neurons were compared with neurons from the L4 dorsal root ganglion (SNL4) and L5 ganglion (SNL5) after L5 and L6 spinal nerve ligation. Number of neurons for each group is shown in the *bars*. ** $P < 0.01$, *** $P < 0.001$.

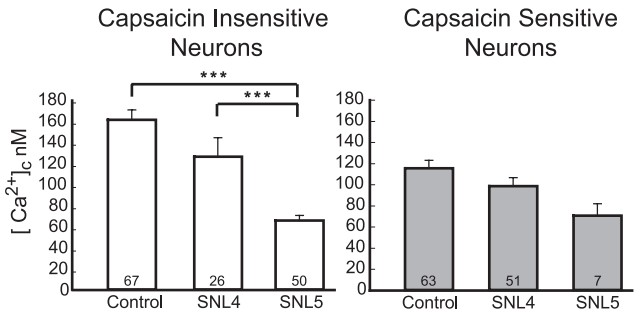

Fig. 9. Injury decreases resting cytosolic calcium concentration ($[Ca^{2+}]_c$) in capsaicin-insensitive sensory neurons. Control neurons were compared to neurons from the L4 dorsal root ganglion (SNL4) and L5 ganglion (SNL5) after L5 and L6 spinal nerve ligation. There was no significant main effect of injury (analysis of variance) in capsaicin-sensitive neurons. Number of neurons for each group is shown in the *bars*. *** $P < 0.001$.

the influence of injury did not reach significance ($P = 0.077$), due in part to the small number of SNL L5 neurons that were sensitive to capsaicin.

Discussion

Although calcium is the principal signaling pathway controlling cellular processes of sensory neurons, there has been only minimal examination of effects of peripheral nerve injury on $[Ca^{2+}]_c$. In this study, we demonstrate that axonal injury depresses resting calcium concentrations. This decrease is greater in directly axotomized neurons than in adjacent neurons exposed to degenerating axonal segments. The influence of injury is not uniform across neuronal types but is dominant in units presumed to have a nonnociceptive modality, as indicated by large somatic diameter and insensitivity to capsaicin.

We are not aware of other reports of resting $[Ca^{2+}]_c$ in painful neuropathic conditions, except for experimental diabetes mellitus. Although some studies of streptozotocin-induced diabetes have found increased resting $[Ca^{2+}]_c$,[23,24] the relevance to traumatic or inflammatory neuropathy is uncertain. For example, diabetes is associated with increased voltage-activated calcium currents in the DRG neuronal membrane,[31] whereas we have determined that traumatic neuropathy decreases these currents.[12,13] Also, there are contrasting reports that show unchanged sensory neuronal resting $[Ca^{2+}]_c$ in streptozotocin-induced diabetic rats[32–34] and in a spontaneously diabetic rat strain.[35]

Response of $[Ca^{2+}]_c$ to Capsaicin

Our intent in examining capsaicin response was to categorize cells according to sensory modality.[17] We also confirmed an EC_{50} for $[Ca^{2+}]_c$ response to capsaicin in the 55–72 nM range,[36,37] which is much less than the 350–728 nM necessary to produce a membrane current.[38–41] Sensory neurons express the capsaicin receptor TRPV1 on endoplasmic reticulum,[42] so release of calcium stored in endoplasmic reticulum[43] may explain the responses to low capsaicin concentrations we observed. Supporting our belief that the $[Ca^{2+}]_c$ response to capsaicin is a valid indicator of nociceptive modality, we noted a higher response rate in small cells, which tend to be nociceptors. Further, the response rate was substantially decreased in the axotomized neurons of the L5 DRG after SNL but increased in L4, consistent with immunohistochemical observations of TRPV1 expression.[44,45] A generally higher resting $[Ca^{2+}]_c$ in control cells tested with capsaicin compared with those categorized only by size may be due to an unexplained effect of capsaicin on ionophore calibration. Nonetheless, injury effects between groups persisted in cells treated this way.

Technique of Measuring $[Ca^{2+}]_c$

Determination of resting $[Ca^{2+}]_c$ is highly sensitive to technical details. The use of a ratiometric indicator such as Fura-2 largely corrects for variations in dye loading, bleaching, or differences in fluorescent signal due to cell thickness. We have noted substantial variability between neurons in R_{min}, R_{max}, and β. This requires the determination of calibration parameters for each neuron if $[Ca^{2+}]_c$ is to be compared between cells, although averaged calibration parameters may be adequate if repeated measures for a given cell are compared (*e.g.*, response to an agonist).

Technical differences may in part explain the wide range of resting $[Ca^{2+}]_c$ reported for sensory neurons that spans from 60 to 207 nM.[24,36,46–48] Further factors may influence measured $[Ca^{2+}]_c$, however, including choice of K_d, age of the animal,[47] and variability in neuronal size and sensory modality of the sample population, as we have shown in the current study. Our finding of higher resting $[Ca^{2+}]_c$ in large-diameter sensory neurons than in small neurons contrasts with previous findings in mice[32,49] but is in accord with findings in rats.[24]

Potential Mechanisms of Depressed $[Ca^{2+}]_c$ after Injury

Cytosolic calcium is regulated by the balanced actions of various membrane processes that acquire, store, and expel calcium. Although calcium entry through voltage-gated calcium channels does not influence $[Ca^{2+}]_c$ of the inactive neuron,[50] influx of calcium through voltage-independent channels regulated by calcium stores (capacitative calcium entry) is active at rest and sensitive to resting membrane potential.[50,51] Uptake into endoplasmic reticulum *via* the sarcoplasmic or endoplasmic reticulum calcium adenosine triphosphatase pump and expulsion of calcium from the cell by the plasma membrane calcium adenosine triphosphatase pump both regulate resting $[Ca^{2+}]_c$,[52] and mitochondria also sequester calcium in the inactive neuron.[53] In most sensory neu-

rons, the plasmalemmal Na^+–Ca^{2+} exchanger is constitutively active such that removal of extracellular Na^+ increases resting $[Ca^{2+}]_c$.[54] The decrease in resting $[Ca^{2+}]_c$ after peripheral nerve injury may result from a disturbance in any of these regulatory processes, but these have not been examined.

Withdrawal of target-derived neurotrophins may be an upstream trigger for depression of $[Ca^{2+}]_c$, because nerve growth factor,[55] brain-derived neurotrophic factor, and neurotrophin-3[56] support resting $[Ca^{2+}]_c$. However, nerve growth factor in the ligated L5 DRG recovers after 2 days,[57] and brain-derived neurotrophic factor expression is increased after nerve injury.[58,59] An alternate cause of depressed resting $[Ca^{2+}]_c$ may be decreased neuronal firing, because activation of neurons increases resting $[Ca^{2+}]_c$.[60,61] Decreased afferent traffic due to axotomy or disuse of the limb combined with injury-induced depression of voltage-activated calcium currents[12,13] may decrease the cytoplasmic calcium load. Although spontaneous activity develops in injured DRG neurons,[8,62] this is typically at very low rates.[63,64] The normal or higher levels of resting $[Ca^{2+}]_c$ in neurons from SNL rats that lacked hyperalgesia might reflect particularly high levels of spontaneous activity in these subjects. Differences we noted between putative nociceptive and low-threshold neurons may likewise be due to differences in neuronal activity between these modality categories.

Possible Consequences of Decreased $[Ca^{2+}]_c$

A role for decreased resting $[Ca^{2+}]_c$ in generation of neuropathic pain is supported by our observation of a normal or increased resting $[Ca^{2+}]_c$ in a small number of rats lacking hyperalgesia after SNL. Although the functional consequence of dynamic $[Ca^{2+}]_c$ transients is well established, the role of resting $[Ca^{2+}]_c$ has been less studied. In various neuronal tissues, decreased $[Ca^{2+}]_c$ precipitates cell loss, including programed cell death by apoptosis.[60,61,65] DRG neuronal loss has been noted as a feature of neuropathy after SNL,[66,67] and induced cell activity may prevent cell loss after axotomy in the central nervous system, presumably by increasing resting $[Ca^{2+}]_c$.[68] Therefore, the decrease in resting $[Ca^{2+}]_c$ we have observed may directly lead to loss of sensory neurons after injury.

A second role of resting $[Ca^{2+}]_c$ is control of responsiveness to ligand stimulation. Increased resting $[Ca^{2+}]_c$ attenuates receptor-triggered calcium signaling in lymphocytes[69] and central nervous system microglia.[70] In DRG neurons, increased $[Ca^{2+}]_c$ inactivates responses to capsaicin.[37,38,71] In contrast, responsiveness to heat is potentiated when $[Ca^{2+}]_c$ is increased by capsaicin or a calcium ionophore,[72] but this is not found after $[Ca^{2+}]_c$ increase by membrane depolarization.[73] Depressed resting $[Ca^{2+}]_c$, as we have measured in sensory neurons after injury, may thus modulate the transduction of membrane receptor activation into intracellular calcium signals for a broad range of ligands and stimuli, including catecholamines, purines, pH, and inflammatory mediators such as bradykinin, complement, and cytokines. Altered expression of receptors may have a competing influence, however, as in the injury-induced changes in capsaicin responsiveness measured in this study. Low resting $[Ca^{2+}]_c$ may itself produce complex genetic effects, increasing expression of inducible nitric oxide synthase in chondrocytes while depressing synthesis of cyclooxygenase II,[74] and increasing the expression of sodium channels in the surface membrane of adrenal chromaffin cells.[75]

Finally, enzymatic signaling cascades sensitive to calcium, such as calmodulin, phosphatases, and protein kinases A and C, modulate neuronal activity. Through regulation of calcium-activated K^+ channels[76] and hyperpolarization-activated cation channels,[77] resting $[Ca^{2+}]_c$ controls membrane excitability and neuronal activity. Calcium/calmodulin-dependent protein kinase II has particularly diverse phosphorylation targets and may be an important pathway generating functional and genetic changes from shifts in resting $[Ca^{2+}]_c$.[78]

Conclusion

Our findings indicate that nerve injury associated with hyperalgesia depresses resting $[Ca^{2+}]_c$ in sensory neurons. This change is most evident in nonnociceptive axotomized neurons of L5 after SNL, which supports a central role of this neuronal group in the genesis of neuropathic pain.[9] However, these observations do not eliminate the possibly critical contributions of functional changes in calcium signaling of neurons that remain in continuity to their receptive fields, since we also identified decreased resting $[Ca^{2+}]_c$ in large L4 neurons after SNL.

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