Axotomy Depletes Intracellular Calcium Stores in Primary Sensory Neurons

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Background: The cellular mechanisms of neuropathic pain are inadequately understood. Previous investigations have revealed disrupted Ca2+ signaling in primary sensory neurons after injury. The authors examined the effect of injury on intracellular Ca2+ stores of the endoplasmic reticulum, which critically regulate the Ca2+ signal and neuronal function.

Methods: Intracellular Ca2+ levels were measured with Fura-2 or mag-Fura-2 microfluorometry in axotomized fifth lumbar (L5) dorsal root ganglion neurons and adjacent L4 neurons isolated from hyperalgesic rats after L5 spinal nerve ligation, compared to neurons from control animals.

Results: Endoplasmic reticulum Ca2+ stores released by the ryosomotropic agonist caffeine were increased by 46% in axotomized small neurons. This effect persisted in Ca2+-free bath solution, which removes the contribution of store-operated membrane Ca2+ channels, and after blockade of the mitocondrial, sarco-endoplasmic Ca2+-ATPase and the plasma membrane Ca2+ ATPase pathways. Ca2+ released by the sarco-endoplasmic Ca2+-ATPase blocker thapsigargin and by the Ca2+-ionophore ionomycin was also diminished by 25% and 41%, respectively. In contrast to control neurons, Ca2+ stores in axotomized neurons were not expanded by neuronal activation by K+ depolarization, and the proportionate rate of refilling by sarco-endoplasmic Ca2+-ATPase was normal. Luminal Ca2+ concentration was also reduced by 38% in axotomized neurons in permeabilized neurons. The adjacent neurons of the L4 dorsal root ganglia showed modest and inconsistent changes after L5 spinal nerve ligation.

Conclusions: Painful nerve injury leads to diminished releasable endoplasmic reticulum Ca2+ stores and a reduced luminal Ca2+ concentration. Depletion of Ca2+ stores may contribute to the pathogenesis of neuropathic pain.

THE primaryafferent neuron is the source of all somatic sensory experience, a target of regional anesthetics, theafferent pathway for pain during surgery, and an origin of abnormal activity in chronic pain. Cytoplasmic Ca2+ is the dominant second messenger in sensory neurons, and it regulates diverse processes, including neuronal differentiation, neurotransmitter release, excitability, gene expression, enzymatic activation, and programmed cell death (apoptosis). We have previously shown that painful nerve injury leads to prolonged disruption of the Ca2+ signal in primary afferent neurons. Specifically, after transection of their peripheral axons, neurons with their cell bodies in the dorsal root ganglia (DRGs) demonstrate a depressed resting cytoplasmic Ca2+ concentration ([Ca2+]c) as well as a diminished temporary increase of [Ca2+]c that follows neuronal activation (the [Ca2+]c transient). These findings may be partially attributable to the decrease in activity-related Ca2+ entry through the plasmalemma caused by axotomy of DRG neurons. However, the cellular machinery that modulates the sensory neuron Ca2+ signal is complex, and its function in the context of injury has not been examined.

Sensory neurons possess channels and pumps that extrude Ca2+ from the cell, sequester Ca2+ into organelles within the cell, and release Ca2+ from these stores. Specifically, the plasma membrane Ca2+-ATPase expels cytoplasmic Ca2+ from the neuron, as does the Na+-Ca2+ exchange pathway. Cytoplasmic Ca2+ is pumped through the action of the sarcoplasmic-endoplasmic reticulum Ca2+-ATPase (SERCA) into high-concentration subcellular reservoirs, predominantly the endoplasmic reticulum (ER), from which it may be released back to the cytoplasm through channels sensitive to ryanodine (the ryanodine receptor [RyR]) or inositol triphosphate (IP3). The amount of Ca2+ accumulated in this store is functionally linked to plasma membrane channels (store-operated Ca2+ channels [SOCs]) that admit Ca2+ into the cytoplasm at a rate inversely related to the store level. Mitochondrial Ca2+ uptake driven by the potential across the mitochondrial inner membrane buffers large Ca2+ loads by uptake when the [Ca2+]c rises above a critical threshold and subsequently releases this Ca2+ as the [Ca2+]c recovers towards baseline levels. All of these processes are tuned by regulatory systems and may be sites at which neuronal injury perturbs the final Ca2+ signal.

The quantity of stored Ca2+ critically regulates Ca2+ signaling in neurons. This parameter dictates the ability of the sensory neuron to receive Ca2+ from the cytoplasm, by which the cell limits excessive [Ca2+]c levels during neuronal activity. Storied Ca2+ may also be released into the cytoplasm through the process of Ca2+ induced Ca2+ release, by which an initial increase in [Ca2+]c activates the RyRs to discharge stored Ca2+ into the cytoplasm, resulting in amplification of [Ca2+]c transients. Thus, the ER is at once both a Ca2+ sink and source, critically shaping the Ca2+ signal.
Disordered homeostasis of the Ca$^{2+}$ store participates in the pathogenesis of various neurologic conditions, such as stroke and Alzheimer disease; as well as painful conditions such as diabetes, human immunodeficiency virus infection, and tissue inflammation.$^{12-14}$ The ER stores may likewise play a central role in the pathogenesis of neuropathic pain. Accordingly, the experiments reported here explored the influence of injury on the size of the intracellular Ca$^{2+}$ store. We employed the spinal nerve ligation (SNL) injury model; this allows separate evaluation of the axotomized neurons of the fifth lumbar (L5) DRG for comparison with the L4 neurons that are not directly injured but have axons that transit the sciatic nerve in the company of degenerating distal segments of the L5 neurons, and are thus exposed to mediators of inflammation.$^{15,16}$ The relative importance of the contribution to pain genesis of these two populations remains unresolved.$^{17}$

**Materials and Methods**

All methods and use of animals were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (Milwaukee, Wisconsin).

**Injury Model**

Male Sprague-Dawley (Taconic Farms Inc., Hudson, NY) rats weighing 160–180 g were subjected to SNL in a manner derived from the original technique.$^{18}$ Rats were anesthetized with 2% isoflurane in oxygen, and the right paravertebral region was exposed. After removal of the L6 transverse process, the L5 and L6 spinal nerves were ligated with 6-0 silk suture and transected distal to the ligature. The fascia was closed with 4-0 resorbable polyglactin suture, and the skin was closed with staples. Control animals received anesthesia, skin incision, and stapling only. After surgery, the rats were returned to their cages and kept under normal housing conditions with access to pellet food and water *ad libitum.*

**Sensory Testing**

Rats underwent sensory testing for hyperalgesic behavior on three different days between 10 and 17 days after surgery, as previously described.$^{1,19}$ Briefly, right plantar skin was mechanically stimulated with a 22-gauge spinal needle with adequate pressure to indent but not penetrate the skin. Whereas control animals respond with only a brief reflexive withdrawal, rats after SNL may display a complex hyperalgesia response that incorporates sustained licking, chewing, grooming, and elevation of the paw. The frequency of hyperalgesia responses was tabulated for each rat. After SNL, only rats that displayed a hyperalgesia-type response after at least 20% of stimuli were used further in this study.

**Neuron Isolation and Plating**

Neurons were dissociated from L4 and L5 DRGs after isoflurane anesthesia and decapitation 21 to 27 days after SNL or skin sham surgery. This interval was chosen because hyperalgesia is fully developed by this time.$^{19}$ DRGs were incubated in 0.0625% trypsin (Sigma Aldrich, St. Louis, MO), 0.0125% DNase (Invitrogen, Carlsbad, CA), and 0.01% blendzyme 2 (Roche Diagnostics, Indianapolis, IN) in Dulbecco modified Eagle’s medium (DMEM)/F12 with glutaMAX (Invitrogen) for 1.5 h, centrifuged, and triturated with fire-polished pipettes in culture medium containing Neural Basal Media A with B27 supplement (Invitrogen), 0.5 mM glutamine, 100 ng/ml nerve growth factor 7S (Alomone Labs, Jerusalem, Israel), and 0.02 mg/ml gentamicin (Invitrogen). Dissociated neurons were plated onto poly-l-lysine coated glass cover slips (Deutsches Spiegelglas; Carolina Biological Supply, Burlington, NC) and maintained at 37°C in humidified 95% air and 5% CO$_2$ for 2 h, and were studied no later than 6 h after harvest.

**Solutions and Agents**

Unless otherwise specified, the bath contained Tyrode’s solution (in mM): NaCl 140, KCl 4, CaCl$_2$ 2, Glucose 10, MgCl$_2$ 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, with an osmolarity of 297–300 mOsm and pH 7.40. In some experiments, a Ca$^{2+}$-free Tyrode’s was used that contained (in mM): NaCl 140, KCl 4, Glucose 10, MgCl$_2$ 2, HEPES 10, and ethylene glycol tetraacetic acid (EGTA) 0.2. In experiments with permeabilized neurons, a bath solution modeled upon intracellular solution was used that contained (in mM): KCl 120, EGTA 5, CaCl$_2$ 2.25, MgCl$_2$ 2, and HEPES 20. Osmolarity was adjusted to 295 with sucrose, and pH was adjusted to 7.40. This clamped Mg$^{2+}$ and Ca$^{2+}$ at physiologic concentrations$^{20}$ of 1.6 mM and 39 mM, respectively, calculated using a web-based EGTA calculator.$^{20}$ Adenosine-triphosphate was omitted to not trigger release of stored Ca$^{2+}$. Agents were obtained as follows: caffeine, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), dimethylsulfoxide, lanthana chloride, thapsigargin from Sigma Aldrich, saponin from Calbiochem EMD (Gibbstown, NJ), Fura-2-AM, mag-Fura-2-AM, ionomycin, and Pluronic F-127 from Invitrogen. Stock solutions of ionomycin, thapsigargin, FCCP, Fura-2-AM, and mag-Fura-2-AM were dissolved in dimethylsulfoxide and subsequently diluted in the relevant bath solution such that final bath concentration of dimethylsulfoxide was 0.1% or less, which has no effect on [Ca$^{2+}$]$_c$ ($n = 20$, data not shown). The 500-μl recording chamber was constantly superfused by a gravity-driven bath flow at a rate of 3 ml/min. Agents were delivered by directed microperfusion through a 500-μm diameter hol-
low quartz fiber 300 μm upstream from the neurons. This flow completely displaced the bath solution, and constant flow was maintained by delivery of bath solution, when specific agents were not being administered, through a computerized valve system. Solution changes were achieved within 200 ms.

**Measurement of Cytoplasmic Ca²⁺ Concentration**

Cover slips holding plated neurons were transferred to a room-temperature, 5 μM solution of Fura-2-AM that contained 0.04% Pluronic F-127 to aid dispersion of the fluorophore. After 30 min, they were washed three times with regular Tyrode’s solution and left in a dark environment for de-esterification for 30 min and then mounted onto the recording chamber. The fluorophore was excited alternately with 340-nm and 380-nm wavelength illumination (150W Xenon, Lambda DG-4; Sutter, Novato, CA), and images were acquired at 510 nm by using a cooled 12-bit digital camera (Coolsnap fx; Photometrics, Tucson, AZ) and inverted microscope (Diaphot 200; Nikon Instruments, Melville, NY) through a 20X or 40X fluor oil-immersion objective. Neurons were visually examined in the brightfield mode, and those showing signs of lysis, crenulation, or superimposed glial cells were excluded. Recordings from each neuron were obtained as separate regions of interest by appropriate software (MetaFluor; Molecular Devices, Downingtown, PA). After background subtraction, the fluorescence ratio R for individual neurons was determined as the intensity of emission during 340-nm excitation (I_340) divided by I_380, on a pixel-by-pixel basis. The calcium concentration was then estimated by the formula [Ca²⁺]_c = K_d · β · (R - R_min)/(R_max - R) where β = (I_380max/I_380min). Values of R_min, R_max, and β were determined by in situ calibrations of 33 neurons from three different preparations as described before and were 0.38, 8.49, and 9.54, respectively, and 224 nm was used as K_d.²¹

**Determination of Ca²⁺ Release**

Release of Ca²⁺ from stores was quantified by measuring the size of the resulting [Ca²⁺]_c transient. Using amplitude to quantify large transients, such as those induced by the action of caffeine, poorly reflects the magnitude of Ca²⁺ release from stores because of the influence of mitochondrial buffering during intervals of high [Ca²⁺]_c. Initial mitochondrial Ca²⁺ uptake depresses the peak [Ca²⁺]_c during a transient and produces a plateau phase thereafter, during which Ca²⁺ is released from mitochondria into the cytoplasm.²² Thus, mitochondrial buffering has reciprocal effects on transient amplitude and duration.²³ Further, we have previously shown that transient area is proportionate to cytoplasmic Ca²⁺ load.² We therefore chose to quantify caffeine-induced transients by calculating the area of the transient.

In other experiments, application of thapsigargin was used to produce transients, for which the amplitude of the rise from baseline to peak [Ca²⁺]_c was measured rather than area, because these transients had a more gradual onset without a clearly discernible beginning and endpoint, unlike those triggered by caffeine. Also, such transients do not reach [Ca²⁺]_c levels that engage mitochondrial buffering, which we estimated from the level of the plateau in large K⁺- (316 ± 124 nM; n = 115) and caffeine-induced transients (310 ± 128 nM, n = 541).²²,²⁴ Amplitude measurement was also used for transients induced by ionomycin; this ionophore to equilibrate [Ca²⁺]_i gradients between subcellular compartments, including cytosol, ER, and mitochondria.²⁵ Calcium was absent from the bath solution during thapsigargin application to remove the contribution of Ca²⁺ entry through SOCCs²⁶ and similarly during ionomycin application to avoid entry of Ca²⁺ through the ionophore.

**Measurement of ER Luminal Ca²⁺ Concentration**

The low-affinity indicator mag-Fura-2-AM was used to measure the Ca²⁺ concentration contained within the lumina of intracellular compartments ([Ca²⁺]_l).²⁷,²⁸ Dye loading and recording procedures were similar to those used with Fura-2-AM, although loading took place at 37°C to maximize compartmentalization of the fluorophore in intracellular organelles.²⁹ Calibration of the mag-Fura-2 signal was performed in situ on 26 neurons from 3 different dissociated DRGs and showed values of [Ca²⁺]_l, and β of 0.32, 3.63, and 13.09. A K_d of 53 μM was used for calculation of [Ca²⁺]_l.³⁰ Attempts using patch-pipette dialysis in protocols requiring removal of cytoplasmic mag-Fura-2 proved ineffective, but successful permeabilization of neurons was achieved by 75- to 105-s bath application of 0.01% saponin.³¹ Cytoplasmic washout of the fluorophore was confirmed by following the decline in both the fluorescence intensities at 340 nm and 380 nm and a concurrent increase in the ratio R. Only traces in which the decline in intensity during 380 nm illumination exceeded 40% were included for analysis.

**Statistical Analysis**

Preliminary studies showed no differences between neurons dissociated from L4 and L5 ganglia, removed from the same control animals, in Ca²⁺ release by caffeine with normal Tyrode’s bath solution (transient area 14.7 ± 11.3 nM · s × 10³ in L4 [n = 19] vs 15.7 ± 11.4 nM · s × 10³ in L5 [n = 37]; P = 0.38). We therefore pooled findings from L4 and L5 neurons as the control group. Neurons characterized by size (large, greater than 54 μm; small, less than 34 μm) were considered separately. Statistical analyses were performed with Statistica (StatSoft Inc, Tulsa, OK). Two-tailed Student t test was used for comparing two means. One-way ANOVA was used to detect the influence of injury group on measured parameters. Consideration of additional factors such as time or pharmacologic interventions was achieved using a two-way ANOVA design. An ANCOVA model was used.
Results

Upon needle stimulation, SNL animals (n = 69) displayed a hyperalgesia response rate of 35 ± 21%, whereas control animals (n = 56) showed a hyperalgesic response 0 ± 1% (P < 0.001). Consistent with our previous findings,1 sensory neuron axotomy depressed resting [Ca2+]c. Specifically, whereas [Ca2+]c was 66 ± 27 nM in control neurons (n = 274), SNL of L5 neurons resulted in a [Ca2+]c of 46 ± 25 nM (n = 157; P < 0.001 vs. control). SNL had no effect on resting [Ca2+]c of adjacent L4 neurons, in which [Ca2+]c was 63 ± 26 nM (n = 110).

Ryanodine Receptor-mediated Ca2+ Release by Caffeine

An estimate of stored Ca2+ may be made by measuring the rise of [Ca2+]c during Ca2+ release from stores triggered by application of a high concentration of caffeine (20 mM), which sensitizes the RyR to Ca2+ to such an extent that resting levels of Ca2+ produce sustained channel opening.32 For caffeine-induced Ca2+ release, we chose to measure transient area rather than amplitude to limit the influence of mitochondrial buffering (see Materials and Methods), and we tested the assumption in control neurons by comparing transient parameters with and without blockade of mitochondrial Ca2+ buffering with FCCP (1 μM).22 Whereas amplitude of the caffeine-induced transient increased by 108% in the presence of FCCP (from 779 ± 807 nM [n = 273] to 1615 ± 2075 nM [n = 198]), area of the transient increased by only 20% (from 18.2 ± 11.1 nM · s × 103 [n = 268] to 21.9 ± 16.6 nM · s × 103 [n = 198]).

To measure stores, caffeine was administered continuously until the [Ca2+]c returned to baseline to assure that the stores were fully emptied, which was confirmed by the observation that reapplication of caffeine 30 s after washout produced no further Ca2+ release (n = 10; data not shown). Sustained application also precluded SERCA function so that this process did not affect the measure of Ca2+ release. Application of caffeine (20 mM) in this fashion produced a rapidly rising transient that included a prolonged plateau phase followed by resolution to a level close to resting [Ca2+]c (fig. 1A). All but 1 of 542 neurons responded with an increase of at least 50% above resting [Ca2+]c.

DRG neurons are a heterogeneous population representing different sensory modalities. Although small neurons are broadly associated with nociceptive sensory modality, neurons with large somata conduct low-threshold sensory information.33,34 In small neurons, the transient areas evoked by caffeine were significantly decreased by axotomy in L5 neurons after SNL, as well as in adjacent L4 neurons (fig. 1B). Caffeine-induced transients had areas in control large neurons that were smaller than in control small neurons (P < 0.001). However, there was no effect of injury on large neuron Ca2+ release measured this way (fig. 1C).

Caffeine release of stored Ca2+ triggers influx of Ca2+ across the plasmalemma through store-operated Ca2+ channels (SOCCs),35 which could be independently influenced by injury. We therefore performed additional experiments in small neurons, eliminating this factor by using Ca2+-free bath solution.36 Under these conditions, transients triggered by caffeine application showed influences of injury that were congruent with the findings above (fig. 1D). The transient area in all groups was less in the absence of bath Ca2+ compared to experiments conducted with bath Ca2+, which likely indicates a contribution by SOCC to the transient generated by caffeine release of Ca2+ stores.35

To further compare Ca2+ stores and injury effect in putative nociceptors versus low-threshold afferents, we examined additional small neurons that were further categorized by sensitivity to capsaicin. Sensitivity to capsaicin is typical of peptidergic polymodal nociceptors, and insensitivity is a characteristic of IB4-binding mechanosensitive nociceptors.37 In a separate preliminary study (data not shown), the EC50 for capsaicin necessary to achieve a 50% increase in [Ca2+]c was 7.9 nM (total n = 175 neurons, used only for this determination). Using 10 nM, 45 of 85 (53%) of control neurons responded to capsaicin by this criterion (fig. 1E), comparable to the typical proportion of nociceptive neurons.38 In control neurons, [Ca2+]c transient areas were significantly larger in capsaicin-responsive cells than in nonresponsive ones (P < 0.001; fig. 1F), as reported previously. SNL decreased the proportion of neurons sensitive to capsaicin in the L4 group (10 of 37, 27%) and especially the L5 group (8 of 53, 15%; P < 0.001), as expected from previous findings that axonal injury causes loss of the capsaicin receptor, transient receptor potential vanilloid-1,39 In both capsaicin-responsive and nonresponsive populations, axotomy reduced the area of the caffeine-induced transient (fig. 1F), indicating loss of Ca2+ stores. Specifically, in capsaicin-responsive neurons, the average area under the transient curve was decreased by 59% in axotomized compared to control neurons, and axotomy of capsaicin-nonresponsive neurons decreased the area by 33%. This indicates that both subgroups of small nociceptive sensory neurons have decreased releasable Ca2+ stores after injury.
**Ca\(^{2+}\)** Release during Inhibition of Sequestration and Extrusion

To examine Ca\(^{2+}\) release in the absence of the buffering by uptake into mitochondria, we applied caffeine (20 mM) during the continued presence of FCCP (1 \(\mu\)M; fig. 2A). Peak of the transient in L4 neurons was not different from controls, while the axotomized L5 neurons decreased by 51% compared to controls (fig. 2B). This indicates that smaller caffeine-induced \([\text{Ca}^{2+}]_c\) transients in axotomized neurons are not due to greater mitochondrial buffering in this neuronal group.

We also eliminated the potential influence of variable Ca\(^{2+}\) reuptake by the SERCA, Ca\(^{2+}\) extrusion via the plasma membrane Ca\(^{2+}\)-ATPase and Ca\(^{2+}\)-Na\(^{+}\) exchange mechanism, and Ca\(^{2+}\) influx by inducing Ca\(^{2+}\) release with caffeine (20 mM) in the context of multiple pharmacological blockers (FCCP, 1 \(\mu\)M; thapsigargin, 1 \(\mu\)M to block SERCA, administered only with and after caffeine to avoid an initial thapsigargin-induced store release; La\(^{3+}\), 1 mM to block plasma membrane Ca\(^{2+}\)-ATPase, Ca\(^{2+}\)-Na\(^{+}\) exchange, and Ca\(^{2+}\) entry pathways, discontinued during caffeine in order to avoid precipitation). This resulted in transients in which an initial increase of \([\text{Ca}^{2+}]_c\) was not followed by recovery (fig. 2C). Injury had a significant effect on the caffeine-induced transient amplitude (fig. 2D), in which the L4 group was un-

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**Fig. 1.** Response of cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) in sensory neurons to application of caffeine (20 mM). (A) Characteristic transients show a rapid peak and sustained plateau, with diminished size after spinal nerve ligation (SNL) in axotomized small neurons of the fifth lumbar (L5) dorsal root ganglion. (B) In small neurons (diameter less than 34 \(\mu\)m), transient area is decreased in L5 neurons compared to control (C). (C) In large neurons, there are no differences between injury groups. (D) When Ca\(^{2+}\) is removed from the bath solution, transients in small neurons are reduced in size compared to Ca\(^{2+}\)-containing solution (panel B), but injury still reduces transient in L5 neurons, as well as in L4 neurons. (E) The frequency of neuronal sensitivity to capsaicin (10 nM) is decreased in L5 neurons after SNL. (F) The area of caffeine-induced transients is less in neurons insensitive to capsaicin than in responsive ones in control neurons but not in L4 or L5 neurons after SNL. Transient area is reduced in L5 neurons compared to control in both capsaicin sensitive and insensitive populations. For panels B–E, one-way ANOVA with Bonferroni post hoc testing was used. For panel F, two-way ANOVA with Bonferroni post hoc testing was used. For all panels, numbers in bars indicate n for number of neurons, brackets above bars connect groups that are significantly different (P < 0.05), and error bars show SD.
Ca²⁺ Release during Extrusion/Sequestration Block

A Mitochondrial Block with FCCP

FCCP (1µM) and Caffeine (20mM)

\([\text{Ca}^2+]_c\) (nM)

C SNL L5

0 1000 2000 3000 4000

B Data Summary

\(\Delta[\text{Ca}^2+]_c\) (nM)

C SNL L4 SNL L5

198 87 87

2500

C Block of Mitochondria, SERCA, PMCA, and Na⁺/Ca²⁺ Exchange

FCCP 1µM

La³⁺ 1mM

\([\text{Ca}^2+]_c\) (nM)

0 1000 2000 3000

D Data Summary

\(\Delta[\text{Ca}^2+]_c\) (nM)

C SNL L4 SNL L5

86 70 109

Ca²⁺ Release by Ionomycin

Application of the Ca²⁺ ionophore ionomycin (1 µM) is an additional technique that releases stored Ca²⁺ without requiring receptor activation and eliminates the influence of mitochondria. A Ca²⁺-free bath solution was used for 3 min before ionomycin application and thereafter to avoid the influence of Ca²⁺ entry through the plasmalemma. Transients showed an exponential resolution that lacked the plateau attributable to mitochondria (fig. 4A). After SNL, ionomycin released more Ca²⁺ into the adjacent L4 neurons, but releasable Ca²⁺ was reduced in axotomized L5 neurons compared to control (fig. 4B).

It is unclear whether ryanodine receptor-sensitive Ca²⁺ stores fully overlap with Ca²⁺ stores sensitive to inositol-1P. For this reason and because receptor-mediated Ca²⁺ release may be influenced by altered expression of the receptor after injury without actually affecting Ca²⁺ stores per se, we also determined the effect of injury on Ca²⁺ stores using techniques that do not rely on receptor activation. Thapsigargin provides such an alternate method by blocking SERCA, which exposes a constitutive Ca²⁺ leak from stores. This effect is dose-related; although a previously published study using 20 nM thapsigargin failed to produce transients in sensory neurons, we observed slow \([\text{Ca}^2+]_c\) transients with application of 1 µM thapsigargin in all neurons (fig. 3A). Full emptying of RyR-sensitive stores by thapsigargin was demonstrated by reduction of the subsequent caffeine-induced Ca²⁺ release transient in control neurons to 165 ± 353 nm · s (n = 20), which is 1% of caffeine-induced Ca²⁺ release reported above. However, thapsigargin-sensitive Ca²⁺ stores encompass more than just the RyR-sensitive pool; a thapsigargin-induced transient (amplitude 23 ± 17 nm, n = 19) persists after caffeine-induced Ca²⁺ release, which constitutes 22% of the thapsigargin-induced transient in the absence of preceding caffeine (107 ± 69, n = 85). An effect of injury is evident in the thapsigargin-sensitive pool; the thapsigargin-induced transient in SNL L5 neurons had a 25% lower transient amplitude than control or SNL L4 neurons (fig. 3B).

The rise of \([\text{Ca}^2+]_c\) during thapsigargin application was reliably a straight line (fig. 3A, inset) indicative of a zero-order process, consistent with the observation that the leak rate from Ca²⁺ stores is independent of \([\text{Ca}^2+]_c\) until the stores are nearly exhausted. To evaluate the rate of constitutive release from thapsigargin-sensitive stores, we measured the slope of this rising phase of the thapsigargin-induced \([\text{Ca}^2+]_c\) transient. Leak rate measured this way showed a tight dependence on total releasable Ca²⁺ content, as indicated by amplitude of the thapsigargin transient (R² = 0.85 for all groups together, P < 0.001; n = 95 neurons, fig. 3C). For this reason, an analysis of covariance (ANCOVA) model was used to examine the average leak rate, which was depressed in SNL L5 neurons compared to control and SNL L4 neurons (fig. 3D). This was attributable to the effect of the depressed releasable Ca²⁺ in the L5 neurons, as demonstrated by the comparable relationship between leak rate and releasable Ca²⁺ in control and L5 neurons (fig. 3C). However, L4 neurons had a slightly depressed relationship of leak versus level of releasable Ca²⁺.
SERCA-dependent Recovery of Ca^{2+} Stores at Rest

One possible reason that Ca^{2+} stores cannot be filled in injured neurons may be dysfunction of the SERCA mechanism that pumps Ca^{2+} into the ER. Repeat application of caffeine (20 ms) produces a diminished Ca^{2+} release until adequate time has elapsed to allow refilling of the stores (fig. 5A). We therefore tested the pace at which neurons in the different groups recovered from caffeine-induced depletion of stored Ca^{2+}. Complete release of stored Ca^{2+} was achieved by sustained application of caffeine (20 ms), which was followed by a 1-, 5-, or 10-min recovery period, and then caffeine was applied again. Each neuron was used to test only one time interval. After 10-min recovery time, the area of the second transient in control neurons had recovered to 79 ± 23% of the first (fig. 5B), and there was no difference in the proportionate rate of recovery after injury, normalized to the initial baseline Ca^{2+} release. We confirmed that replenishment during rest was SERCA-dependent; thapsigargin applied during the refilling interval reduced 10-min recovery to 0.3 ± 1.4% (n = 20). These findings indicate that SERCA function is adequate after neuronal injury to provide recovery to the original baseline level of stores.

Role of Activity in Regulating Ca^{2+} Stores

Ca^{2+} enters sensory neurons predominantly through voltage-gated Ca^{2+} channels during neuronal activity.
After SNL, axotomized L5 neurons receive no afferent activity generated from natural stimulation of the receptive field, and this loss may thereby lead to depleted Ca\(^{2+}\) stores. We therefore examined whether membrane depolarization with K\(^+\), which in turn leads to Ca\(^{2+}\) influx across the plasmalemma and store repletion, might increase Ca\(^{2+}\) stores in axotomized neurons to the levels of control neurons. Priming transients were generated by 5-s application of 50 ms K\(^+\) followed 3 min later by sustained application of 20 ms caffeine. Compared to caffeine-induced transients without prior in vitro activation, caffeine-induced transients that followed activation by K\(^+\) (fig. 6) were larger by 94% in control neurons and by 182% in SNL L4 neurons, whereas activation produced no expansion (0%) of the caffeine-induced transient in axotomized L5 neurons. However, the K\(^+\)-induced transient in SNL L5 neurons was also smaller (13.0 ± 12.6 \times 10^3 \text{nm} \cdot \text{s}, n = 24) than in control neurons (31.4 ± 27.9 \times 10^3 \text{nm} \cdot \text{s}, n = 40) or SNL L4 neurons (44.1 ± 23.5 \times 10^3 \text{nm} \cdot \text{s}, n = 14), indicating that these groups may have received a greater Ca\(^{2+}\) influx and boost in stores than the SNL L5 population. Application of K\(^+\) lasting only 2 s resulted in K\(^+\)-induced transients in control neurons (10.2 ± 7.2 \times 10^3 \text{nm} \cdot \text{s}, n = 30) that were comparable in area to those produced by 5 s K\(^+\) activation in SNL L5 neurons, but these similar Ca\(^{2+}\) loads still showed a greater priming effect in control neurons (28.7 ± 20.1 \times 10^3 \text{nm} \cdot \text{s}, n = 29; 58% greater than baseline caffeine-induced transient) than in axotomized L5 neurons (0%). These observations indicate that the deficit in Ca\(^{2+}\) stores after injury cannot be repaired by activity, and therefore is not due to neuronal disuse.

**Direct Measurement of [Ca\(^{2+}\)]\(_{L}\)**

Pharmacological control of Ca\(^{2+}\) release, sequestration, and extrusion is inevitably imperfect, due to incomplete drug specificity and efficacy. We therefore devised a technique for directly measuring Ca\(^{2+}\) concentration in the subcellular stores using the low-affinity Ca\(^{2+}\) fluorophore mag-Fura-2.\(^{31}\) Although this approach defines the concentration of unbound Ca\(^{2+}\) in the membrane-delimited Ca\(^{2+}\) stores rather than their net mass, it can be assumed that the concentration of free luminal Ca\(^{2+}\) is directly related to total stored Ca\(^{2+}\).

We initially determined [Ca\(^{2+}\)]\(_{L}\) in nonpermeabilized neurons.\(^{41}\) Although such neurons contain mag-Fura-2 in the cytoplasm, the conditions of loading (see Materials and Methods) were optimized to preferentially accumulate mag-Fura-2 in organelle compartments. Under these conditions, calculated [Ca\(^{2+}\)]\(_{L}\) was found to be significantly lower in L4 neurons and especially L5 neurons after SNL compared to control (fig. 7A). Caffeine (20 mM) was applied to the loaded neurons to validate the location of mag-Fura-2 in the stored Ca\(^{2+}\) compartment. This produced a decrease of calculated [Ca\(^{2+}\)]\(_{L}\) by at least 10% in 107 of 131 (82%) of the neurons, although this may be an underestimation resulting from the influences of caffeine.\(^{42}\) If the principal source of fluorescence emission was in fact cytoplasmic rather than compartmentalized mag-Fura-2, release of Ca\(^{2+}\) stores would
Fig. 7. Determination of the Ca\(^{2+}\) concentration in the endoplasmic reticulum (ER) lumen ([Ca\(^{2+}\)]\(_L\)) using the low-affinity fluophore mag-Fura-2. (A) In nonpermeabilized neurons, the estimated [Ca\(^{2+}\)]\(_L\) is lower than control in neurons from the fourth lumbar (L4) dorsal root ganglion after spinal nerve ligation (SNL), and lower in L5 neurons than both other groups. (B) Application of saponin in a bath solution resembling intracellular solution (free [Ca\(^{2+}\]) 39 nm) allows washout of cytoplasmic mag-Fura-2, indicated by a decrease in intensity of fluorescence emission during both 380 nm (I\(_{380}\)) and 440 nm (I\(_{440}\)) excitation. The resulting [Ca\(^{2+}\)]\(_L\) calculated from the I\(_{380}/\)I\(_{440}\) ratio increased to a peak indicative of [Ca\(^{2+}\)]\(_L\). Subsequent application of ionomycin releases Ca\(^{2+}\) from the stores, represented by decreased [Ca\(^{2+}\)]\(_L\). (C) The [Ca\(^{2+}\)]\(_L\) in permeabilized neurons is depressed in axotomized L5 neurons after SNL compared to control. For panels A and C, one-way ANOVA with Bonferroni post hoc testing was used, numbers in bars indicate \(n\) for number of neurons, brackets above bars connect groups that are significantly different (\(P < 0.05\)), and error bars show SD.

ER Luminal Ca\(^{2+}\) Measurement by Mag-Fura-2

A Intact Neurons

B Membrane Permeabilization

C Permeabilized Neurons

Discussion

The somata of sensory neurons are known to be substantially affected by injury of their peripheral axons. Our current findings extend this recognition by showing that a fundamental feature, the intracellular Ca\(^{2+}\) store, is depleted after injury. Specifically, the amount of Ca\(^{2+}\) that can be released from stores through a variety of pathways is diminished after axotomy, and the concentration of Ca\(^{2+}\) in the luminal compartment is also reduced as estimated by direct imaging.

Intracellular Ca\(^{2+}\) stores in neurons reside in several organelles. The best characterized is the ER store, where

As has been done in other studies, we included Mg\(^{2+}\) in the bath to maintain a natural intracellular concentration after permeabilization. However, entry of bath Mg\(^{2+}\) acting on residual cytoplasmic mag-Fura-2 could contribute to our observed increase of [Ca\(^{2+}\)]\(_L\) during permeabilization and bias our determination of [Ca\(^{2+}\)]\(_L\). This is an unlikely explanation, because this would also cause fluorescence intensities to increase during permeabilization, whereas we observed them to decrease. However, to also test this factor, permeabilization was performed in a set of control neurons using Mg\(^{2+}\)-free bath solution, which produced a [Ca\(^{2+}\)]\(_L\) (152 ± 90 \(\mu\)M, \(n = 28\)) that was minimally different from experiments in which the bath solution contained Mg\(^{2+}\) (105 ± 69 \(\mu\)M, \(n = 94\); \(P = 0.16\)).
Ca²⁺ also critically regulates the luminal processes of protein assembly and folding. Additional sites of Ca²⁺ storage include the nuclear envelope and the Golgi apparatus. The contribution of storage in secretory vesicles and so-called calciosomes remains poorly defined. Recent evidence indicates the dominant site for the Ca²⁺ pool is in the ER lumen. Although subcellular localization may play a role in directing released Ca²⁺ to particular targets, current techniques do not allow these stores to be functionally distinguished.

We used standard techniques to identify the magnitude of the Ca²⁺ store. Caffeine freely diffuses through the cell membrane and, by activating the RyR, completely discharges Ca²⁺ from the stores available to this release pathway. Although caffeine interacts with Ca²⁺-sensitive fluorophores, these effects are minor when using ratiometric indicators such as in the current studies. Stored Ca²⁺ measured this way is affected selectively for specific subgroups of neurons. Axotomy by SNL has no effect on caffeine-releasable Ca²⁺ in large neurons that mostly transduce and transmit low-threshold sensory information. However, axotomy substantially depresses caffeine-releasable stores in the small neuron population, especially in capsaicin-sensitive putative nociceptors. Capsaicin nonresponsive small neurons, which probably represent a population of nonpeptidergic, isolectin B4-binding mechanosensitive nociceptors, also have depleted stores after injury, but are less affected than capsaicin sensitive neurons. Loss of Ca²⁺ store in only certain neuron subtypes after axotomy may be due to ER damage selectively in these neurons, as is explored in the companion paper.

Sensory neurons possess additional intracellular Ca²⁺ stores that may be released through activation of an IP₃-sensitive channel. There is not full agreement about whether the Ca²⁺ pools sensitive to IP₃ and caffeine overlap in peripheral sensory neurons, although experiments using sequential activation of these pathways mostly support the view that the pools are one and the same. We did not measure Ca²⁺ release by IP₃ because injury of peripheral sensory neurons directly affects IP₃ signaling and membrane permeant or caged IP₃ compounds are not readily available. We therefore turned to an alternate strategy of discharging stores indiscriminately either by the Ca²⁺ ionophore ionomycin or by constitutive leak from stores during SERCA inhibition by thapsigargin. Observations using both of these techniques support the findings from caffeine experiments and show an axotomy-induced deficit of Ca²⁺ in stores that is independent of the release mechanism.

The Ca²⁺ transient during release of stores is supplemented by Ca²⁺ influx through SOCCs. Therefore, a loss of this capacitative Ca²⁺ entry process due to injury could create the appearance of a deficit in stores measured by release. However, we observed that the depressant effect of axotomy on store release persists in Ca²⁺-free bath solution, so this explanation is unlikely. Also, we considered whether neuronal inactivity might have led to store depletion after axotomy. However, cytoplasmic Ca²⁺ loads provided by activation of voltage-gated Ca²⁺ channels minimally expanded stores in axotomized neurons compared to control neurons. Together, our findings support the conclusion that the capacity of the intracellular Ca²⁺ storage reservoir is compromised after axotomy in sensory neurons.

The amount of stored Ca²⁺ is dictated by the concentration of Ca²⁺ in the store, [Ca²⁺]ᵢ, the bound Ca²⁺ in equilibrium with this free fraction, and the extent of the compartment. Therefore, although mag-Fura-2 microfluorimetry reflects [Ca²⁺]ᵢ, it cannot indicate the total mass of stored Ca²⁺ per se. Although the concentration of the free fraction should be proportionate with the total Ca²⁺ store, this method cannot account for changes in the amount or performance of various Ca²⁺-binding proteins in the compartment, which could alter also the mass of stored Ca²⁺ without changing [Ca²⁺]ᵢ. Our findings with mag-Fura-2 reveal a decrease in resting [Ca²⁺]ᵢ associated with axotomy. Caffeine produces a decrease in [Ca²⁺]ᵢ measured by mag-Fura-2; therefore, it is likely that the mag-Fura-2 technique quantifies the state of the same Ca²⁺ pool as does caffeine-induced release. The combined results of these two approaches point to a model in which axotomy decreases the ability of the storage compartment, predominantly the ER, to concentrate Ca²⁺ within its lumen. This level of [Ca²⁺]ᵢ is set by the dynamic balance between SERCA and constitutive Ca²⁺ leakage from the ER. Although resting axotomized neurons show no delay in fractional recovery of Ca²⁺ stores back to their baseline after release, this is nonetheless compatible with compromised SERCA function because baseline store levels are comparably depleted. We showed that the relationship between constitutive Ca²⁺ leak rate and Ca²⁺ store level is normal in axotomized neurons, which points to a normal leak pathway. This leaves compromised SERCA function as the cause of decreased [Ca²⁺]ᵢ, which could be the result of altered regulation by kinases. Our data do not reveal whether injury also diminishes the extent of the stores, which is addressed in the companion paper.

The effect of painful conditions on neuronal Ca²⁺ stores has previously been examined only in models of diabetes mellitus. Those studies found that Ca²⁺ transients induced by ionomycin or caffeine are decreased in diabetic animals, similar to our observations after peripheral nerve trauma. However, a substantially different pathogenic mechanism in diabetes is suggested by differences in other aspects of the Ca²⁺ signaling pathway in sensory neurons, including an increased resting [Ca²⁺]ᵢ and prolonged activity-induced

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transients,\(^{61,62,64}\) which contrast with decreased resting [Ca\(^{2+}\)]\(_l\) and decreased activity-induced transients after axotomy.\(^{1,2,65}\) Like diabetes, painful peripheral inflammation is associated with elevated resting [Ca\(^{2+}\)]\(_i\) and transients in DRG neurons,\(^{14}\) but the state of the Ca\(^{2+}\) stores in this condition has not been determined.

The SNL model provides the opportunity to separately evaluate neurons in the L5 DRG that are axotomized versus the adjacent L4 neurons. After SNL, features of Ca\(^{2+}\) storage and release in these two populations are divergent. In contrast to axotomized L5 neurons, ionomycin release of stored Ca\(^{2+}\) is elevated in L4 neurons, although this may represent increased release from the mitochondrial component of stored Ca\(^{2+}\) released. The L4 neurons also show an expanded Ca\(^{2+}\) storage capacity, as shown by a supranormal level of releasable Ca\(^{2+}\) after cytoplasmic Ca\(^{2+}\) loading. Finally, whereas axotomy diminishes Ca\(^{2+}\) release by caffeine or thapsigargin and decreases [Ca\(^{2+}\)]\(_i\), measured by mag-Fura-2 in peripheral L5 neurons, these are unaffected in the L4 population. These divergent findings may result from the difference in predominant influences of SNL on the L5 versus L4 neurons. Whereas the SNL results in axotomy of the L5 population, the peripheral axons of the adjacent L4 neurons remain intact but are exposed to inflammatory mediators triggered by Wallerian degeneration of detached L5 axonal segments.\(^{15,16,66}\) Other studies have shown divergent influences on Ca\(^{2+}\) handling in inflammation compared to axotomy. Specifically, models of inflammatory pain reveal increased activity-induced Ca\(^{2+}\) transients in DRG neurons,\(^{14}\) whereas the opposite occurs after axotomy.\(^{2}\) Our current findings also suggest that Ca\(^{2+}\) stores are also differentially regulated by axotomy and inflammation. A degree of inconsistency in our L4 findings may be attributable to a variable admixture of axotomy among the L4 neuronal population following L5 SNL.\(^{67}\)

Disruption of the level of Ca\(^{2+}\) stores in neuronal subcellular compartments is a fundamental pathogenic factor in a variety of diseases, including ischemia, acquired immunodeficiency syndrome, and Alzheimer disease.\(^{6}\) In addition to storing Ca\(^{2+}\), the ER is the site assembly and posttranslational modification of proteins by glycosylation and folding. Depletion of luminal Ca\(^{2+}\) triggers highly conserved stress responses involving accumulation of unfolded protein, global suppression of protein synthesis, and activation of a variety of transcription factors, resulting in neuronal dysfunction and death.\(^{68}\) For instance, Ca\(^{2+}\) store depletion by caffeine or thapsigargin exposure suppresses protein synthesis and induces neuronal apoptosis independent of increased [Ca\(^{2+}\)]\(_i\).\(^ {69,70}\) The current findings suggest that similar processes may take place in sensory neurons after peripheral nerve injury and that they may contribute to long-term changes associated with chronic pain.

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