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PAINFUL NERVE INJURY DECREASES SARCO-ENDOPLASMIC RETICULUM CA^{2+}-ATPASE ACTIVITY IN AXOTOMIZED SENSORY NEURONS

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Abstract—The sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a critical pathway by which sensory neurons sequester cytosolic Ca^{2+} and thereby maintain intracellular Ca^{2+} homeostasis. We have previously demonstrated decreased intraluminal endoplasmic reticulum Ca^{2+} concentration in traumatized sensory neurons. Here we examine SERCA function in dissociated sensory neurons using Fura-2 fluorometry. Blocking SERCA with thapsigargin (1 μM) increased resting $[Ca^{2+}]_c$ and prolonged recovery (τ) from transients induced by neuronal activation (elevated bath K^+), demonstrating SERCA contributes to control of resting $[Ca^{2+}]_c$ and recovery from transient $[Ca^{2+}]_c$ elevation. To evaluate SERCA in isolation, plasma membrane Ca^{2+} ATPase was blocked with pH 8.8 bath solution and mitochondrial buffering was avoided by keeping transients small (⩽400 nM). Neurons axotomized by spinal nerve ligation (SNL) showed a slowed rate of transient recovery compared to control neurons, representing diminished SERCA function, whereas neighboring non-axotomized neurons from SNL animals were unaffected. Injury did not affect SERCA function in large neurons. Repeated depolarization prolonged transient recovery, showing that neuronal activation inhibits SERCA function. These findings suggest that injury-induced loss of SERCA function in small sensory neurons may contribute to the generation of pain following peripheral nerve injury.

Key words: sarco-endoplasmic reticulum Ca^{2+}-ATPase, Ca^{2+} homeostasis, neuropathic pain, endoplasmic reticulum, sensory neuron.

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Abbreviations: ANOVA, analysis of variance; $[Ca^{2+}]_c$, cytoplasmic Ca^{2+} concentration; $[Ca^{2+}]_L$, endoplasmic reticulum lumen Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L4, fourth lumbar; L5, fifth lumbar; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SNL, spinal nerve ligation; SOCC, store-operated Ca^{2+} channel; SPCA, secretory pathway Ca^{2+}/Mn^{2+}-ATPases; TG, thapsigargin; TTA-P2, 3,5-dichloro-N-[1-(2,2-dimethyltetrahudro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide; VGCC, voltage-gated Ca^{2+} channel.

INTRODUCTION

Intracellular Ca^{2+} levels ($[Ca^{2+}]_c$) regulate neuronal excitability, release of neurotransmitters, cell differentiation and apoptosis (Ghosh and Greenberg, 1995; Mata and Sepulveda, 2005). Thus, dysregulation of $[Ca^{2+}]_c$ signaling leads to diverse neuropathological conditions (Fernyhough and Calcutt, 2010; Gleichmann and Mattson, 2011; Stutzmann and Mattson, 2011). Neuronal activation triggers an influx of Ca^{2+} through voltage-gated Ca^{2+} channels (VGCCs), requiring buffering, sequestration, and ultimately Ca^{2+} efflux to reestablish Ca^{2+} homeostasis. Efflux pathways include the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) (Benham et al., 1992; Suzuki et al., 2002; Usachev et al., 2002; Guerini et al., 2005; Lytton, 2007), while sequestration of Ca^{2+} into organelle compartments is achieved by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), mitochondrial Ca^{2+} uniporter, and secretory pathway Ca^{2+}/Mn^{2+}-ATPases (SPCA) (Fierro et al., 1998; Wuytack et al., 2002; Verkhratsky, 2004, 2005; Usachev et al., 2006; Sepulveda et al., 2007; Brini and Carafoli, 2009).

We have previously demonstrated that painful peripheral nerve injury is associated with aberrant Ca^{2+} signaling in peripheral sensory neurons after axotomy, including reduced resting $[Ca^{2+}]_c$ (Fuchs et al., 2005), diminished activity-induced transients (Fuchs et al., 2007), and accelerated store-operated Ca^{2+} channel (SOCC) function (Gemes et al., 2011). Also, releasable Ca^{2+} stored in the endoplasmic reticulum (ER) is depleted and the concentration of Ca^{2+} in the ER lumen ($[Ca^{2+}]_L$) is depressed by injury in small sensory neurons (Rigaud et al., 2009). The $[Ca^{2+}]_L$ is set by the dynamic balance between SERCA and constitutive Ca^{2+} leakage from the ER through poorly defined channels (Camello et al., 2002). Our prior finding of normal Ca^{2+} release channel function after injury (Rigaud et al., 2009) suggests compromised SERCA performance as the cause of decreased $[Ca^{2+}]_L$ in injured neurons.

SERCA is the principal high-affinity sequestration pathway for Ca^{2+} that enters during neuronal activation (Verkhratsky, 2005), and accounts for the most of intracellular uptake during low amplitude $[Ca^{2+}]_c$ transients that are insufficient to engage mitochondrial buffering (Usachev et al., 2006). Normal SERCA function is required to maintain intracellular stores that provide releasable Ca^{2+}, which in turn regulates

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neuronal excitability (Gemes et al., 2011). SERCA dysfunction contributes to numerous pathological conditions, including diabetic axonopathy (Zherebitskaya et al., 2012), Ca^{2+} overload during neuronal ischemia (Larsen et al., 2005; Henrich and Buckler, 2008), age-associated neuronal degeneration (Pottorf et al., 2000a,b), excitotoxicity (Fernandes et al., 2008), ER stress and apoptosis (Mengesdorf et al., 2001; Verkhratsky, 2004; Gallego-Sandin et al., 2011). Little is known about the modulation of SERCA function after peripheral nerve injury or its role in chronic pain.

To characterize the effect of neuronal trauma, we have measured SERCA function in rats subjected to spinal nerve ligation (SNL) (Kim and Chung, 1992; Hogan et al., 2004), which provides neuronal populations that are axotomized (in the fifth lumbar dorsal root ganglion – L5 DRG), versus the neighboring fourth DRG (L4) neurons that are intact but exposed to inflammation caused by degeneration of the detached distal L5 fiber segments (Gold, 2000). To isolate SERCA, we blocked PMCA, which is the main efflux pathway in neurons (Benham et al., 1992; Usachev et al., 2002).

EXPERIMENTAL PROCEDURES

Animals

All methods and use of animals were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Taconic Farms Inc., Hudson, NY, USA) were housed individually in a room maintained at 22 ± 0.5 °C and constant humidity (60 ± 15%) with an alternating 12-h-light–dark cycle. Food and water were available *ad libitum* throughout the experiments.

Injury model

Rats weighing 150–180 g were subjected to SNL modified from the original technique (Kim and Chung, 1992). Specifically, rats were anesthetized with 2% isoflurane in oxygen and the right paravertebral region was exposed. The L6 transverse process was removed, after which the L5 and L6 spinal nerves were ligated with 6-0 silk suture and transected distal to the ligature. To minimize non-neural injury, no muscle was removed, muscles and intertransverse fascia were incised only at the site of the two ligations, and articular processes were not removed. The muscular fascia was closed with 4-0 resorbable polyglactin sutures and the skin closed with staples. Control animals received skin incision and closure only. After surgery, rats were returned to their cages and kept under normal housing conditions with access to pellet food and water *ad lib*.

Sensory testing

We measured the incidence of a pattern of hyperalgesic behavior that we have previously documented to be associated with conditioned place avoidance (Hogan et al., 2004; Wu et al., 2010). Briefly, on three different days between 10 and 17 days after surgery, right plantar skin was touched (10 stimuli/test) with a 22-G spinal needle with adequate pressure to indent but not penetrate the skin. Whereas control animals respond with only a brief reflexive withdrawal, rats following SNL may display a complex hyperalgesia response that includes licking, chewing, grooming and sustained elevation of the paw. The average frequency of hyperalgesia responses over the 3 testing days 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 rapidly harvested from L4 and L5 DRGs during isoflurane anesthesia and decapitation 21–28 days after SNL or skin sham surgery. This interval was chosen since hyperalgesia is fully developed by this time (Hogan et al., 2004). Ganglia were incubated in 0.5 mg/ml Liberase TM (Roche, Indianapolis, IN, USA) in DMEM/F12 with glutaMAX (Life Technologies, Grand Island, NY, USA) for 30 min at 37 °C, followed with 1 mg/ml trypsin (Sigma–Aldrich, St. Louis, MO, USA) and 150 Kunitz units/ml DNase (Sigma–Aldrich) for another 10 min. After addition of 0.1% trypsin inhibitor (Type II, Sigma–Aldrich), tissues were centrifuged, lightly triturated in neural basal media (1X) (Life Technologies) containing 2% (v:v) B27 supplement (50x) (Life Technologies), 0.5 mM glutamine (Sigma–Aldrich), 0.05 mg/ml gentamicin (Life Technologies) and 10 ng/ml nerve growth factor 7S (Alomone Labs Ltd., Jerusalem, Israel). Cells were then plated onto poly-L-lysine (70–150 kDa, Sigma–Aldrich) coated glass cover slips (Deutsches Spiegelglas, Carolina Biological Supply, Burlington, NC, USA) and incubated at 37 °C in humidified 95% air and 5% CO_2 for 2 h and were studied 3–8 h after dissociation.

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, HEPES 10, with an osmolarity of 297–300 mOsm and pH 7.40. In some experiments, a modified Tyrode's was used that contained 0.25 mM $CaCl_2$ to generate transient amplitude to smaller than 400 nM after PMCA blockade. To block PMCA, HEPES was replaced by Trizma (10 mM) in regular Tyrode's with adjusted pH at 8.8 (Duman et al., 2008).

Fura-2-AM was obtained from Invitrogen (Carlsbad, CA, USA), and the SERCA blocker thapsigargin (TG) and SOCC blocker lanthanum chloride from Sigma–Aldrich (St. Louis, MO, USA). Selective VGCC subtype antagonists including nimodipine (5 μM) to block L-type current (Wu et al., 2008), ω-conotoxin GVIA (GVIA, 200 nM) to block N-type current (Randall and Tsien, 1995; McCallum et al., 2011), ω-conotoxin MVIIC (MVIIC, 200 nM) to block both N- and P/Q-type current (Randall and Tsien, 1995; McCallum et al., 2011), and 3,5-dichloro-N-[1-(2,2-dimethyltetrahudro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2; 1 μM; a kind gift from the Merck Research Laboratories, West Point, PA, USA), which is a potent and selective blocker of T-type current in rat sensory neurons (Choe et al., 2011). Stock solutions of TG, nimodipine, TTA-P2 and Fura-2-AM were dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted in the relevant bath solution such that final bath concentration of DMSO was 0.2% or less, which does not effect $[Ca^{2+}]_c$ (Avery and Johnston, 1997; Gemes et al., 2011). Other VGCC antagonist stock solutions were dissolved in water. The 500-μl recording chamber was superfused by gravity-driven flow at a rate of 3 ml/min. Agents were delivered by directed microperfusion controlled by a computerized valve system (ALA Scientific Instruments, Farmingdale, NY, USA) through a 500-μm diameter hollow quartz fiber 300-μm upstream from the neurons. This flow completely displaced the bath solution, and constant flow was maintained through this microperfusion