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Increased thrombospondin-4 after nerve injury mediates disruption of intracellular calcium signaling in primary sensory neurons

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ABSTRACT

Painful nerve injury disrupts Ca^{2+} signaling in primary sensory neurons by elevating plasma membrane Ca^{2+}-ATPase (PMCA) function and depressing sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) function, which decreases endoplasmic reticulum (ER) Ca^{2+} stores and stimulates store-operated Ca^{2+} entry (SOCE). The extracellular matrix glycoprotein thrombospondin-4 (TSP4), which is increased after painful nerve injury, decreases Ca^{2+} current (I_{Ca}) through high-voltage–activated Ca^{2+} channels and increases I_{Ca} through low-voltage–activated Ca^{2+} channels in dorsal root ganglion neurons, which are events similar to the effect of nerve injury. We therefore examined whether TSP4 plays a critical role in injury-induced disruption of intracellular Ca^{2+} signaling. We found that TSP4 increases PMCA activity, inhibits SERCA, depletes ER Ca^{2+} stores, and enhances store-operated Ca^{2+} influx. Injury-induced changes of SERCA and PMCA function are attenuated in TSP4 knock-out mice. Effects of TSP4 on intracellular Ca^{2+} signaling are attenuated in voltage-gated Ca^{2+} channel $\alpha_2\delta_1$ subunit ($Ca_v\alpha_2\delta_1$) conditional knock-out mice and are also Protein Kinase C (PKC) signaling dependent. These findings suggest that TSP4 elevation may contribute to the pathogenesis of chronic pain following nerve injury by disrupting intracellular Ca^{2+} signaling via interacting with the $Ca_v\alpha_2\delta_1$ and the subsequent PKC signaling pathway. Controlling TSP4 mediated intracellular Ca^{2+} signaling in peripheral sensory neurons may be a target for analgesic drug development for neuropathic pain.

1. Introduction

Neuronal cytoplasmic Ca^{2+} is the essential regulator of numerous physiological functions, such as excitation, synaptic transmission, synaptic plasticity, and neuronal differentiation and survival (Catterall et al., 2013; Gemes et al., 2011; Paschen, 2001).

When neurons are activated, free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) increases due to Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) or ligand-gated Ca^{2+} channels on the plasma membrane. This may be supplemented by release of Ca^{2+} from intracellular stores upon activation of ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3R) on the membrane of endoplasmic reticulum (ER). In addition, Ca^{2+} can enter neurons through channels that are indirectly activated by the depletion of ER Ca^{2+} stores in the process known as store-operated Ca^{2+} entry (SOCE) (Gemes et al., 2011). After neuronal activation, elevated $[Ca^{2+}]_i$ recovers back to resting levels by Ca^{2+} extrusion through the plasma membrane Ca^{2+}-ATPase (PMCA) (Duncan et al., 2013) and Na^+-Ca^{2+} exchanger (NCX), and by Ca^{2+} sequestration into the ER by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Gemes et al., 2012). We have previously observed that, after nerve injury, sensory neurons of the dorsal root ganglia (DRG) develop decreased resting $[Ca^{2+}]_i$ (Fuchs et al., 2005), depressed Ca^{2+} influx through

Abbreviations: TSP4, thrombospondin-4; $Ca_v\alpha_2\delta_1$, voltage-gated calcium channel $\alpha_2\delta_1$ subunit; $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; DRG, dorsal root ganglion; VGCCs, Voltage-gated calcium channels; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; PMCA, plasma membrane Ca^{2+}-ATPase; NCX, Na^+-Ca^{2+} exchanger; ER, endoplasmic reticulum; SOCE, Ca^{2+} stores and stimulates store-operated Ca^{2+} entry; RyRs, ryanodine receptors; IP_3R, inositol 1,4,5-trisphosphate receptors; KO, knock-out; CKO, conditional knock-out; Wildtype, WT; SNL, spinal nerve ligation; PKC, protein kinase C; PKA, protein kinase A; IB4, isolectin B4; ERK, Extracellular signal-regulated kinase; GBP, gabapentin.

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VGCCs (Hogan et al., 2000), and decreased SERCA function (Duncan et al., 2013), as well as elevated Ca^{2+} clearance from the cytoplasm by PMCA function (Gemes et al., 2012) and enhanced Ca^{2+} influx through SOCE due to depletion of ER Ca^{2+} stores (Gemes et al., 2011). However, the pathological effector driving these events is undefined.

Thrombospondins (TSP) are a family of large oligomeric, extracellular matrix glycoproteins that mediate interactions between cells and interactions of cells with underlying matrix components (Adams, 2001; Risher and Eroglu, 2012). The TSP family consists of five members (TSP 1–5) divided into two subgroups (TSP1/2 *vs*. TSP3/4/5) according to their functional domains (Risher and Eroglu, 2012). TSP4 is expressed by astrocytes and neurons, in which it can promote neurite outgrowth and synaptogenesis (Arber and Caroni, 1995; Eroglu et al., 2009; Pan et al., 2015). Recently, TSP4 has been found to be a factor contributing to neuropathic pain (Crosby et al., 2014; Kim et al., 2012). Specifically, TSP4 gene and protein expression is elevated in DRGs after peripheral nerve injury, and intrathecal administration of TSP4 protein amplifies excitatory presynaptic transmission in the dorsal horn via promoting excitatory synaptogenesis by binding to its receptor, voltage-gated Ca^{2+} $\alpha_2\delta_1$ subunit ($Ca_v\alpha_2\delta_1$) (Kim et al., 2012; Pan et al., 2015; Park et al., 2016). However, specific cellular and molecular mechanisms by which TSP4 acts are still unclear.

Various observations support the view that actions of TSP4 may contribute to the pathological effects of axotomy in sensory neurons. Specifically, application of TSP4 *in vitro* reproduces the *in vivo* effects of nerve injury on VGCCs (Hogan et al., 2000; Pan et al., 2016). Additionally, expression of TSP4 protein is elevated in axotomized sensory neurons following peripheral nerve injury (Pan et al., 2015), which suggests that TSP4 may have an important role in regulating intracellular signaling. Here, we test our hypothesis that elevated TSP4 after nerve injury leads to depletion of ER Ca^{2+} stores and disordered function of PMCA, SERCA, and SOCE in primary sensory neurons.

2. Materials and methods

2.1. Animals

Male adult mice (129S1/SvImJ) and *TSP4* gene knock-out (KO) mice (B6.129P2-Thbs4*tm1Dgen*/J) were obtained from The Jackson Laboratory. The $Ca_v\alpha_2\delta_1$ advillin conditional knock-out (CKO) mice were generated by crossing mice with Cre-recombinase expression in advillin positive cells to 129/sv background mice with exon 6 of the $Ca_v\alpha_2\delta_1$ gene (MGI ID: 88295) floxed with loxP sites (Park et al., 2016). $CKO^{Adv-Cre+/-}$ mice have conditionally knocked out $Ca_v\alpha_2\delta_1$ in sensory neurons. $CKO^{Adv-Cre-/-}$ littermates mice were used as wildtype (WT) control. Male Sprague Dawley rats weighing 170–200 g used in some experiments were obtained from The Taconic Biosciences. All animals were housed in a room with a 12/12 h day/night cycle and free access to food and water. Male mice were used for initial studies and female mice were used to confirm major findings. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (AUA00001809 for experiments on mice and AUA00002752 for experiments on rats). Those animals were maintained and used according to the NIH *Guide for the Care and Use of Laboratory Animals*, and in compliance with federal, state, and local laws. DRGs from mice and rats were rapidly harvested following deep isoflurane anesthesia and decapitation.

2.2. Neuron isolation and plating

DRGs from mice or rats were rapidly harvested following deep isoflurane anesthesia and decapitation. Ganglia were placed in a 35 mm dish containing Ca^{2+}/Mg^{2+}-free, cold HBBS (Life Technologies) and cut into four to six pieces that were incubated in in 0.01% blendzyme 2 (Roche Diagnostics, Indianapolis, IN) for 26 min followed by incubation in 0.25% trypsin (Sigma Aldrich, St. Louis, MO) and 0.125% DNAse (Sigma) for 30 min, both dissolved in Dulbecco's modified Eagle's medium (DMEM)/F12 with glutaMAX (Invitrogen, Carlsbad, CA). After exposure to 0.1% trypsin inhibitor and centrifugation, the pellet was gently triturated in culture medium containing Neural Basal Media A with B27 supplement (Invitrogen), 0.5 mM glutamine, 10 ng/ml nerve growth factor 7 S (Alomone Labs, Jerusalem, Israel) and 0.02 mg/ml gentamicin (Invitrogen). Dissociated neurons were plated onto poly-L-lysine coated glass cover slips (DeutschesSpiegelglas, 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 8 h after harvest.

2.3. Measurement of cytoplasmic Ca^{2+} concentration

Measurement of $[Ca^{2+}]_i$ was performed following our previously published protocols (Duncan et al., 2013; Gemes et al., 2012). In brief, unless otherwise specified, regular Tyrode's solution (in mM: NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 10 with an osmolarity of 297–300 mOsm and pH 7.40) was used to bathe the neurons. Stock solution of Fura-2-AM (Invitrogen) was dissolved in DMSO and subsequently diluted in the relevant bath solution such that final bath concentration of DMSO was 0.2% or less, which does not affect $[Ca^{2+}]_i$ (Gemes et al., 2011). 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 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 pathway by delivery of bath solution when specific agents were not being administered. Dye imaging shows that solution changes were achieved within 200 ms.

Neurons plated on cover slips were exposed to Fura-2-AM (5 μM) at room temperature in a solution that contained 2% bovine albumin to aid dispersion of the fluorophore. After 30 min, they were washed 3 times with regular Tyrode's solution, given 30 min for de-esterification, and then mounted in the recording chamber. Neurons were first examined under bright field illumination, and those showing signs of lysis, crenulation or superimposed glial cells were excluded. To determine $[Ca^{2+}]_i$, the fluorophore was excited alternately with 340 nm and 380 nm wavelength illumination (150 W Xenon, Lambda DG-4, Sutter, Novato, CA), and images were acquired at 510 nm using a cooled 12-bit digital camera (Coolsnapfx, Photometrics, Tucson, AZ) and inverted microscope (Diaphot 200, Nikon Instruments, Melville, NY) through a 20X objective. Recordings from each neuron were obtained as separate regions (MetaFluor, Molecular Devices, Downingtown, PA) at a rate of 3 Hz. 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, and $[Ca^{2+}]_i$ was estimated by the formula $K_d \cdot \beta \cdot (R-R_{min})/(R_{max}-R)$ where $\beta = (I_{380max})/(I_{380min})$. Values of R_{min}, R_{max} and β were determined by *in-situ* calibration and were 0.38, 8.49 and 9.54, and K_d was 224 nm. Only neurons with stable baseline R traces were further evaluated. Traces were analyzed using Axograph X 1.1 (Axograph Scientific, Sydney, Australia).