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Store-operated calcium entry compensates fast ER calcium loss in resting hippocampal neurons

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ABSTRACT

The endoplasmic reticulum (ER) acts as a dynamic calcium store and is involved in the generation of specific patterns of calcium signals in neurons. Calcium is mobilized from the ER store by multiple signaling cascades, and neuronal activity is known to regulate ER calcium levels. We asked how neurons regulate ER calcium levels in the resting state. Direct ER calcium imaging showed that ER calcium was lost quite rapidly from the somatic and dendritic ER when resting neurons were transiently kept under calcium-free conditions. Interestingly, free ER and free cytosolic calcium was lost continuously across the plasma membrane and was not held back in the cytosol, implying the presence of a prominent calcium influx mechanism to maintain ER calcium levels at rest. When neurons were treated acutely with inhibitors of store-operated calcium entry (SOCE), an immediate decline in ER calcium levels was observed. This continuous SOCE-like calcium entry did not require the activation of a signaling cascade, but was rather a steady-state phenomenon. The SOCE-like mechanism maintains medium–high ER calcium levels at rest and is essential for balanced resting calcium levels in the ER and cytosol.

1. Introduction

In neurons, the influx of free calcium ions (Ca^{2+}) has a strong excitatory effect and acts as a fast messenger of multiple neuronal signaling cascades [1–4]. The mechanisms of regulating calcium influx to the cytosol are well investigated [3,5,6]. In contrast, homeostatic flow of free calcium is barely understood [7]. At rest, neurons show a reasonably stable level of 40–100 nM free cytosolic calcium [8–11]. In neurons, influx of free calcium from the extracellular space generally depends on neuronal activation because it is mediated by calcium-permeable ion channels such as voltage-dependent calcium channels or ionotropic glutamate receptors [5,8]. In addition, the cytosolic calcium concentration is maintained by the sarco-/endoplasmic reticulum calcium ATPase (SERCA), which actively pumps calcium into the ER. SERCA counteracts a passive ER calcium efflux, the ER calcium leakage, and ensures a resting ER calcium level in the range of 60–400 μM [7,12–14]. The ER calcium leak is mediated by leak channels, one of which was recently identified as the protein-conducting complex Sec61 [15–17]. Emptying the intracellular calcium stores is generally linked to the activation of store-operated calcium channels in the plasma membrane. These ion channels mediate entry of calcium from the extracellular space. This store-operated calcium entry (SOCE) or capacitative calcium entry (CCE) is a ubiquitous mechanism and is well described in non-neuronal cells [6,18–21]. The activation of SOCE involves the stromal interaction molecule (STIM), which senses and coordinates regulated calcium release from the ER. STIM proteins trigger SOCE and interact with ion channels in the plasma membrane [6,22–24]. Ion channels attributed to SOCE comprise Orai channels [25–27] and transient receptor potential channels (TrpCs) [28–30].

Although the inducible cascades of ER calcium store operation have been well investigated in non-neuronal cells, the mechanisms by which cells, and neurons in particular, regulate ER calcium levels at rest [3,11,31] is still unclear, because calcium signals are most frequently monitored in the cytosol rather than in the calcium stores directly. The involvement of the SOCE molecule STIM2 in resting calcium homeostasis has been reported for a HeLa cell line and evaluated using extensive siRNA manipulations [32]. Using a STIM2$^{-/-}$ full ko mouse line and cytosolic calcium imaging, it has been shown that STIM2 regulates ER store replenishment in cortical neurons [33]. Recently, it was reported that cerebellar Purkinje neurons use STIM1 to regulate calcium homeostasis [31]. In these experiments,

Abbreviations: CES, carboxylesterase; ER, endoplasmic reticulum; SERCA, sarco-/endoplasmic reticulum calcium ATPase; SOCE, store-operated calcium entry; TED, targeted-esterase induced dye loading.

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Hartmann et al. observed that a rise in cytosolic calcium by activation of metabotropic glutamate receptors is no longer detectable when neurons were kept transiently in a calcium-free perfusion solution, indicating that the ER calcium store is emptied under calcium-free conditions and implying strongly that resting calcium influx is needed to maintain high ER calcium levels. In the present study targeted-esterase-induced dye loading (TED) [34–36] was used to monitor ER calcium fluxes directly. ER calcium was found to leak continuously from the ER. This calcium is not pumped back to the ER from the cytosol, but instead leaves the cell quantitatively across the plasma membrane. The ER calcium loss is then compensated by a continuous resting calcium influx with SOCE-like properties. Acute blockade of this mechanism causes an immediate drop of ER calcium levels. In summary, this study demonstrates a resting SOCE-like calcium influx in resting neurons that balances the resting level of free calcium in the cytosol and the ER lumen.

2. Materials and methods

2.1. TED vector constructs

New TED vector constructs are based on the original mCES2 vector [35] containing mouse carboxylesterase 2 (CES2; Refseq BC015290). To optimize the ER translocation process, $mCES2^{OPT}$ was generated by cloning the CES2 core sequence (without the signal peptide and the ER retention and retrieval signal) into the vector pCMV-myc-ER (Life technologies). The construct carries a carboxyterminal c-Myc-epitope tag and an ER retention and retrieval consensus motif (HKDEL*). To generate a luminal red fluorescent CES2 fusion protein (RedCES2), the red fluorescent protein TagRFP-T [37] was introduced between the signal peptide cleavage site and mCES2 via XhoI restriction of the vector $mCES2^{OPT}$. For lentiviral expression, RedCES2 was cloned into a lentiviral expression vector based on FUGW [38]. In addition, the RedCES2 sequence was subcloned into the vector FCK, a lentiviral vector containing a neuron-specific calmodulin-dependent protein kinase II promoter element [39]. In a further approach, a linker region coding for the amino acid sequence GGSGGSGG, flanked by SalI and XhoI restriction sites was inserted between TagRFP-T and $mCES2^{OPT}$. A brief outline of RedCES2 has been presented elsewhere [7] and the TED method was described [36].

2.2. Expression and Western blot analysis of TED vectors

Transfected TED constructs were expressed in HeLa cells for 2–3 days. Total cellular protein was extracted in lysis buffer (1% Igepal, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 2 mM Na-orthovanadate (Sigma), 1 mM NaF, 10 mM Na-pyrophosphate and protease inhibitors). Proteins were detected after Western blotting on PVDF membranes (BioRad) with primary mouse anti-c-Myc (1:3000; Santa Cruz; 9E10) or rabbit anti-Esterase (1:5000, Abcam), and secondary goat anti mouse or goat anti-rabbit HRP-coupled antibodies (1:10,000; Jackson immuno research), and ECL substrates (GE Healthcare).

2.3. Lentivirus production

Lentiviral particles were produced in HEK293T cells. Lentiviral expression vectors were co-transfected with the pseudotyping vector pMD2.G and the packaging vector pCMVΔR8.91 [40]. Lentiviral particles were separated from the supernatant by ultracentrifugation, and stored at −80 °C in (in mM) 50 Tris–HCl, pH 7.8, 130 NaCl, 10 KCl, 5 $MgCl_2$.

2.4. Hippocampal neurons

All experiments were performed in accordance with European Union guidelines, as approved by the institutional animal care and utilization committee. Hippocampal cells from CD1 mice of either sex were prepared as described recently [36]. Briefly, hippocampi of new born mice were removed bilaterally and collected in Hank's buffered saline solution (HBSS). Trypsin (Worthington) was added to a final concentration of 0.1% and the tissue incubated for 15 min at 37 °C. The protease digestion was stopped with 0.1% Trypsin inhibitor (Sigma). After 4 steps of trituration in Neurobasal/B27 medium (Life Technologies), cells were plated on poly-L-lysine-coated glass coverslips (Marienfeld) in Neurobasal, 1× B27, 0.5% penicillin/streptomycin, 1% Glutamax, and 1× N2 supplement (all Life Technologies) and cultured at 37 °C under an atmosphere of 5% CO_2. For TED imaging, neurons were transduced with lentiviral TED vectors before seeding onto coverslips. Calcium imaging experiments were performed after 9–16 days in vitro (DIV).

2.5. Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Reverse transcription, primer selection and qPCR reactions used to amplify cDNA encoding for mouse Orai1, Orai2 and Orai3 were carried out as described earlier [41,42] with minor modifications. Primers, PCR conditions and corresponding accession numbers are given in Table S1. RNA was prepared from AraC-treated hippocampal cultures with help of INNUprep RNA Mini Kit (Analytic Jena). qPCR reactions were run on a Lightcycler 1.5 (Roche) using the Luminaris HiGreen qPCR Master Mix. Offline analysis to calculate efficiency-controlled relative expression levels or absolute copy numbers was carried out according to Rasmussen. Intron-spanning primers were selected with Oligo 6.0 software (MedProbe) and PCR conditions, primer concentration and $MgCl_2$ concentration were optimized as described. Reactions were performed in glass capillaries in a volume of 20 μl. PCR products were analyzed by gel electrophoresis, melting curve analysis and control PCR reactions.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2015.04.002

2.6. Immunocytochemistry

Hippocampal neurons were fixed with 4% paraformaldehyde/PBS, pH 7.4 for 15 min. Antibody incubation was performed in PBS containing 10% BSA, 0.3% Triton X100, 0.1% Tween 20. The following primary antibodies were used: mouse anti-c-Myc (1/100, Santa Cruz); rabbit anti-vGlut1 (1/2000, Synaptic Systems), guinea pig anti-vGat (1/400, Synaptic Systems), chicken anti-Map2 (1/2000, Abcam), rabbit anti-Homer1 (1/400, Synaptic Systems), rabbit anti-Calnexin (1/1000, Enzo), rabbit anti-Calreticulin (1/1000, Pierce), chicken anti-Neurofilament heavy chain (1/10.000, Millipore), rabbit anti-Tau (1:1000, Sigma), rabbit anti-STIM1 (1:300, Cell Signaling), and rabbit anti-STIM2 (1:133, Cell Signaling). The following secondary antibodies were used at a concentration of 0.5 μg/ml: donkey anti-guinea pig Cy5, donkey anti-chicken Dylight 649, goat anti-mouse Cy3, donkey anti-rabbit Dylight 488 (Jackson Immuno Research), goat anti-chicken Alexa 488 (Life Technologies). The specimens were embedded in Aqua polymount (Polysciences).

2.7. Confocal laser scanning microscopy and image processing

Images were acquired with an IX81 microscope combined with an Olympus FV1000 confocal laser scanning system, a FVD10 SPD spectral detector and diode lasers of 405 nm, 473 nm, 559 nm, and 635 nm using an Olympus UPLSAPO60x objective (oil, numerical aperture, NA, 1.35). Pinhole setting represented one Airy disk.

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