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Store-operated calcium entry modulates neuronal network activity in a model of chronic epilepsy

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ABSTRACT

Store-operated Ca^{2+} entry (SOCE) over the plasma membrane is activated by depletion of intracellular Ca^{2+} stores and has only recently been shown to play a role in CNS processes like synaptic plasticity. However, the direct effect of SOCE on the excitability of neuronal networks *in vitro* and *in vivo* has never been determined. We confirmed the presence of SOCE and the expression of the calcium sensors STIM1 and STIM2, which convey information about the calcium load of the stores to channel proteins at the plasma membrane, in neurons and astrocytes. Inhibition of SOCE by pharmacological agents 2-APB and ML-9 reduced the steady-state neuronal Ca^{2+} concentration, reduced network activity, and increased synchrony of primary neuronal cultures grown on multi-electrode arrays, which prompted us to elucidate the relative expression of STIM proteins in conditions of pathologic excitability. Both proteins were increased in brains of chronic epileptic rodents and strongly expressed in hippocampal specimens from medial temporal lobe epilepsy patients. Pharmacologic inhibition of SOCE in chronic epileptic hippocampal slices suppressed interictal spikes and rhythmized epileptic burst activity. Our results indicate that SOCE modulates the activity of neuronal networks *in vitro* and *in vivo* and delineates SOCE as a potential drug target.

Introduction

Ca^{2+} transients control a vast array of cellular functions from short-term responses such as contraction and secretion to long-term regulation of cell growth and proliferation. Cytosolic Ca^{2+} increases in response to activation of cell-surface receptors and subsequent generation of the second messenger inositol 1,4,5-trisphosphate (IP3), which interacts with its receptors (IP3R1-3) on the membrane of the endoplasmic reticulum (ER). Opening of these IP3R Ca^{2+} channels releases luminally-stored Ca^{2+} from the ER. Depletion of ER Ca^{2+} stores results in activation of store-operated channels (SOCs) at the plasma membrane, mediating capacitative or store-operated Ca^{2+} entry (SOCE) from the extracellular space followed by removal of cytosolic Ca^{2+} and replenishment of luminal Ca^{2+} through sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA). Based on electrophysiological and molecular properties, two main types of SOCs were proposed: the highly Ca^{2+}-selective I_{CRAC} currents mediated by the ORAI family of proteins carrying the highly Ca^{2+}-selective I_{CRAC} currents (Feske et al., 2006; Vig et al., 2006), and non-selective Ca^{2+} permeable TRPC (C-type transient receptor potential) channels (Huang et al., 2006). The Ca^{2+} sensor that conveys information about the Ca^{2+} load of the ER lumen to SOCs is stromal interaction molecule 1 (STIM1). STIM1 senses luminal Ca^{2+} concentration via an amino-terminal EF hand Ca^{2+}-binding domain and in response to store depletion rearranges into punctate structures close to

Abbreviations: SOCE, Store-operated calcium entry; STIM, Stromal interaction molecule; 2-APB, 2-aminoethoxydiphenyl borate; IP3, Inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; ER, Endoplasmic reticulum; SOC, Store-operated channels; ICRAC, Calcium-release activated calcium current; TRPC, C-type transient receptor potential; NMDA, *N*-Methyl-D-aspartate; PDL, Poly-D-lysine; MEA, Multi-electrode array; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; FAM, Fluorescein amidite; TAMRA, 5-carboxytetramethylrhodamine; HPRT, Hypoxanthine phosphoribosyltransferase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RIPA, Radioimmunoprecipitation assay buffer; PBS-T, Phosphate buffered saline-tween; HBSS, Hanks' Balanced Salt solution; EGTA, Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; PFA, Paraformaldehyde; aCSF, Artificial cerebrospinal fluid; FCS, Fetal calf serum; PBS, Phosphate buffered saline; GFAP, Glial fibrillary acidic protein; ANOVA, Analysis of variance; DG, Dentate gyrus; KA, Kainic acid; MEF, Mouse embryonic fibroblast; TG, Thapsigargin; CTRL, Control; SE, Status epilepticus; WT, Wild type; KO, Knock out.

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the plasma membrane while still remaining in the ER. STIM1 then activates members of the ORAI family of Ca^{2+}-influx channels through direct interaction (Liou et al., 2005; Park et al., 2009; Roos et al., 2005), resulting in Ca^{2+} entry by I_{CRAC} into the cell. As a third element SERCA was shown to co-assemble into STIM1-ORAI1 punctae facilitating the quick shuttling of entering Ca^{2+} into the ER (Manjarrés et al., 2010). In contrast to STIM1, the function of STIM2 is still less clear. STIM2 has been proposed to act as an important sensor of basal ER Ca^{2+} levels and was claimed to be the main regulator of resting ER Ca^{2+} levels in non-excitable HeLa cells (Brandman et al., 2007) and in neurons (Gruszczynska-Biegala et al., 2011).

The role of SOCE and specifically the ER Ca^{2+} sensor STIM1 is best defined in non-excitable cells like immune cells, where it mediates rapid responses such as mast cell degranulation as well as long-term responses that involve new gene transcription (Feske, 2007). However, some effort has been made to elucidate the role of STIM proteins and SOCE also in excitable cells, especially neurons. STIM1 is expressed in cultured neurons and in brain sections with the most prominent expression in cell bodies and dendrites of pyramidal neurons and in Purkinje and granule neurons of the cerebellum (Klejman et al., 2009). STIM1 expression increases during *in vitro* differentiation to relatively high and stable levels in mature neuronal cultures and was found to be present in fractions enriched for postsynaptic densities, suggesting that it may function at or near synapses (Keil et al., 2010). STIM1 and Orai1 were also confirmed in sensory neurons at both the transcript and protein levels (Gemes et al., 2011). Others hardly detected any STIM1 mRNA in pure primary hippocampal neurons of mice isolated by laser capture microscopy and obtained only very weak STIM1 signal from brain lysates in immunoblot analyses (Berna-Erro et al., 2009) and suggested that STIM2 is the main regulator of SOCE in the brain. However, in a parallel work, we compared absolute copy numbers of STIM1 and STIM2 mRNA in hippocampal neurons and showed that STIM2 transcripts are only about twofold more abundant than STIM1 in laser-dissected hippocampal neurons (Gruszczynska-Biegala et al., 2011).

In summary, it appears clear that STIM proteins are present in the brain whereas their role in neuronal function is still uncertain. Blocking of SOCE with Lanthanum attenuated spontaneous Ca^{2+} transients in synaptic boutons, which are important for short-term synaptic plasticity and may also contribute to long-term plasticity (Emptage et al., 2001). Inhibition of SOCE with 2-aminoethoxydiphenyl borate (2-APB) and SKF-96365 in hippocampal slice preparations accelerated the decay of NMDA-induced Ca^{2+} transients without affecting their peak amplitude, and attenuated tetanus-induced dendritic Ca^{2+} accumulation and long-term potentiation at Schaffer collateral-CA1 synapses (Baba et al., 2003) suggesting a link between SOCE and neuroplasticity. Finally, a SOCE influx pathway was also demonstrated in bag cell neurons of *Aplysia* (Kachoei et al., 2006) and in flight neurons of *Drosophila melanogaster* (Agrawal et al., 2010). Blockade of SOCE increased neuronal excitability in dorsal root ganglion neurons (Gemes et al., 2011). Recently, STIM1 was shown to directly suppress depolarization-induced opening of the voltage-gated Ca^{2+} channel $Ca_V1.2$ by binding to the C terminus of this channel, which led to acute inhibition of gating and long-term internalization of the channel from the membrane (Park et al., 2010; Wang et al., 2010).

In this contribution, we tried to pharmacologically elucidate the function of SOCE in the nervous system by the use of dissociated cortical cultures grown on multi-electrode arrays (Otto et al., 2003), which allows a highly sensitive and reproducible assessment of network activity. These data hinted to a role of SOCE in network activity, which is an important determinant of epilepsy. We therefore studied the expression of STIM1 and STIM2 in tissues from chronic epileptic rats and humans and the function of SOCE in a model of chronic epileptiform activity in organotypic hippocampal slice cultures. These experiments showed that STIM proteins are upregulated in conditions of chronic excitability and suggested that SOCE modulates the activity of neuronal networks, which delineates SOCE as a potential drug target in the treatment of chronic epilepsy.

Materials and methods

Cell culture

For the analysis of network activity, cryopreserved primary dissociated cortical cultures of the embryonic rat (embryonic day 18, E18, QBM Cell Science) were employed (Otto et al., 2003). After thawing, the cells were plated at a final density of 10^5 cells on PDL-/laminin-precoated MEAs or coverslips. Neuronal cultures were incubated in a humidified atmosphere (5% CO_2/95% air) at 37 °C. For analysis of STIM1 expression, freshly prepared pure primary embryonic cortical neurons (embryonic day 18, E18) and postnatal astrocytes (postnatal day 0/1, P0/1) were plated on PDL-/laminin-coated or PDL only-coated coverslips. For preparation of astrocytes, P0-1 Wistar rat pups were anesthetized and decapitated, the brain was removed and the neocortex dissected for each brain hemisphere sparing the hippocampi. Meninges were removed and tissue fragments were crudely fragmented. Cell separation was achieved by trituration and by filtration through sterile nylon gauze with a pore size of 60 μm. Astrocytes were cultivated in presence of DMEM supplemented with 10% FBS in a humidified atmosphere (10% CO_2/95% air) at 37 °C with medium renewal every 2–4 days. For removal of non-astroglial contaminations, confluent astrocyte cultures were incubated on a shaker over night, followed by medium renewal. Pure astrocyte cultures were cultivated in the presence of serum-free, chemically defined medium for the generation of astrocyte-conditioned medium. For the preparation of cortical neurons, E18 Wistar rat pups were decapitated, the brain removed, and neocortex tissue prepared as described for the preparation of astrocytes. Cell separation was achieved by trypsinizing the tissue, followed by trituration and by filtration through sterile nylon gauze with a pore size of 30 μm. Cortical neurons were cultivated in the presence of astrocyte-conditioned medium in a humidified atmosphere (10% CO_2/95% air) at 37 °C.

Extracellular microelectrode recordings and signal analysis

Extracellular microelectrode recordings and signal analysis were performed as described (Otto et al., 2003). Network activity was recorded on MEAs (Multi Channel Systems) with 64 titanium nitride electrodes (30 μm diameter and 200 μm spacing) at 37 °C using sterile conditions. Signals from all 64 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MC-Rack (Multi Channel Systems). Spike and burst detection was performed offline using a specialized software (SPANNER 2.0, Result, Germany).

Whole body panel

For relative quantitation of STIM1 and STIM2 mRNA levels in human tissues, a TaqMan™ real-time PCR assay was employed on a 7900 HT Sequence Detection system (Applied Biosystems) according to the manufacturer's protocols. For first strand cDNA synthesis, 85 μg of total RNA was incubated for 1.5 h at 37 °C with 2 U/μl Omniscript reverse transcriptase (Qiagen) in the supplied buffer plus 9.5 μM random hexamer primer, 0.5 mM per dNTP, and 3000 U RnaseOut (Invitrogen) in a final volume of 680 μl. The resulting cDNA was diluted 1:10 with water and directly used in PCR (5 μl per reaction). PCR primers and probes were: STIM1 forward: 5′-CTGACGGAGCCACAGCAT; reverse: 5′-CACTCATGTGGAGGGAGGAC, FAM™/TAMRA™-labeled probe 5′-TCTCAGAGGGATTTGACCCATTCCG, and STIM2 forward: 5′-ACTGGCTCTGCCGCAACT; reverse: 5′-GCATGGTGGACTCAGTGACA, probe 5′-ATAGCCCGGCGCTCATGACAGAT. The real-time PCR thermal protocol was set to 2 min at 50 °C, followed by 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To normalize the amount of cDNA per assay, the expression of reference genes hypoxanthine phosphoribosyltransferase, gapdh, and