Visualization of localized store-operated calcium entry in mouse astrocytes. Close proximity to the endoplasmic reticulum

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Unloading of endoplasmic reticulum (ER) Ca\(^{2+}\) stores activates influx of extracellular Ca\(^{2+}\) channels (SOCs) in the plasma membrane (PM) of most cells, including astrocytes. A key unresolved issue concerning SOC function is their spatial relationship to ER Ca\(^{2+}\) stores. Here, using high resolution imaging with the membrane-associated Ca\(^{2+}\) indicator, FFP-18, it is shown that store-operated Ca\(^{2+}\) entry (SOCE) in primary cultured mouse cortical astrocytes occurs at plasma membrane–ER junctions. In the absence of extracellular Ca\(^{2+}\), depletion of ER Ca\(^{2+}\) stores using cyclopiazonic acid, an ER Ca\(^{2+}\)-ATPase inhibitor, and caffeine transiently increases the sub-plasma-membrane Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{SPM}}\)) within a restricted space between the plasma membrane and adjacent ER. Restoration of extracellular Ca\(^{2+}\) causes localized Ca\(^{2+}\) influx that first increases [Ca\(^{2+}\)]\(_{\text{SPM}}\) in the same restricted regions and then, with a delay, in ER-free regions. Antisense knockdown of the TRPC1 gene, proposed to encode endogenous SOCs, markedly reduces SOCE measured with Fura-2. High resolution immunocytochemistry with anti-TRPC1 antibody reveals that these TRPC-encoded SOCs are confined to the PM microdomains adjacent to the underlying ‘junctional’ ER. Thus, Ca\(^{2+}\) entry through TRPC-encoded SOCs is closely linked, not only functionally, but also structurally, to the ER Ca\(^{2+}\) stores. (Resubmitted 11 February 2005; accepted 23 February 2005; first published online 24 February 2005)

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Astrocytes play a complex role in the CNS: they not only provide structural and metabolic support for neurones, but also actively participate in intracellular signalling (Araque et al. 1999). The dynamic bi-directional form of communication between astrocytes and neurones depends mainly on Ca\(^{2+}\) oscillations and waves (Pasti et al. 1997) supported by localized Ca\(^{2+}\) release from the endoplasmic reticulum (ER) and extracellular Ca\(^{2+}\) entry (Yagodin et al. 1995). The spatial and temporal regulation of Ca\(^{2+}\) signalling in astrocytes is therefore critical (Blaustein & Golovina, 2001). Recent findings indicate that Ca\(^{2+}\) entry through ‘store-operated’ Ca\(^{2+}\) channels (SOCs) in the plasma membrane (PM) appears to be essential to sustain such Ca\(^{2+}\) oscillations (Pizzo et al. 2001; Sergeeva et al. 2003). This store-depletion-activated Ca\(^{2+}\) influx, known as ‘capacitative’ or store-operated Ca\(^{2+}\) entry (SOCE), is the primary mode of regulated Ca\(^{2+}\) entry in electrically non-excitable cells (Putney, 1990; Berridge, 1995).

Substantial evidence indicates that mammalian SOCs are formed by members of a family of seven proteins (TRPC1–TRPC7) that are homologous to the Drosophila transient receptor potential (trp) protein involved in phototransduction (Harteneck et al. 2000; Clapham et al. 2001; Montell, 2001). In particular, four of these proteins, TRPC1, TRPC2, TRPC4 and TRPC5, may form subunits of the endogenous SOCs activated solely by Ca\(^{2+}\) store depletion, while TRPC3, TRPC6 and TRPC7 can be activated by inositol trisphosphate (IP\(_3\)) and/or diacylglycerol and may not be store dependent (Harteneck et al. 2000). The involvement of TRPCs in SOCE in astrocytes, however, is not clear (Pizzo et al. 2001; Grimaldi et al. 2003). Two general mechanisms for opening SOCs have been proposed (Irvine, 1990; Berridge, 1995). One involves a diffusible messenger, presumed to be released from the ER during Ca\(^{2+}\) store depletion, that might open SOCs (Randriamampita & Tsien, 1993; Trepakova et al. 2000). Alternatively, direct interaction between the ER IP\(_3\) receptor (IP\(_3\)R) Ca\(^{2+}\) release channels and plasma membrane SOCs, as a result of store depletion, may trigger SOC opening (Irvine, 1990; Berridge, 1995; Boulay et al. 1999). Such interaction is supported by coimmunoprecipitation of TRPCs and IP\(_3\)R (Boulay et al. 1999). The structural integrity of plasma membrane–‘junctional’ ER units and the spatial

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relationship between SOCs and the ER Ca\(^{2+}\) stores may play an important role in regulating Ca\(^{2+}\) influx. Both SOCE models imply that the PM microdomains containing SOCs are situated in close proximity to the ER. Direct information is, however, lacking about the precise location of SOCs relative to the ER and whether TRPCs contribute to SOCE in astrocytes. These issues are addressed in the present study.

To directly visualize SOCE high spatial resolution imaging with the membrane-associated Ca\(^{2+}\) indicator FFP-18 was employed. FFP-18 is a lipophilic analogue of Fura-2 (Vorndran et al. 1995), which is incorporated into the inner face of the PM (Davies & Hallett, 1996, 1998). FFP-18 has been successfully used to monitor rapid changes in the sub-plasma-membrane Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{PM}}\)) in a variety of cell types (Etter et al. 1994, 1996; Graier et al. 1998; Davies & Hallett, 1998; Chadborn et al. 2002). Using FFP-18 imaging and high resolution immunocytochemistry with anti-TRPC antibody, it is demonstrated here that SOCE signals and encoded by TRPC genes SOCs in cortical astrocytes localize to the PM microdomains adjacent to the underlying 'junctional' ER.

**Methods**

**Astrocyte cultures**

All experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Primary cultured cortical astrocytes were prepared from the brains of 17- to 18-day-old C57BL/6J mouse embryos, as described previously (Golovina et al. 2003); mice were killed by cervical dislocation and fetuses were removed. Cerebral cortices from four to eight fetal mice were separated from the meninges and the hippocampus. The cortices were placed in culture medium (Dulbecco’s modified Eagle’s medium (DMEM/F12 with 10% fetal bovine serum (FBS), penicillin G (50 U ml\(^{-1}\)), and streptomycin (50 µg ml\(^{-1}\)) and were mechanically dissociated by sequential passage through 80 µm and 10 µm nylon mesh to give a single cell suspension. The dissociated cells were plated on either poly-L-lysine-coated 25 mm glass coverslips for use in fluorescence microscope experiments or on coverslips with a lettered grid for counting. The cells were characterized as protoplasmic (type 1) astroglial cells (Bambrick et al. 1996). Experiments were performed on subconfluent cultures on days 8–14 in vitro.

**Calcium imaging**

Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) and [Ca\(^{2+}\)]\(_{\text{PM}}\) were measured with, respectively, Fura-2 and Fura-piperazine-C\(_{12}\)H\(_{25}\) (FFP-18; TEFLabs, Austin, TX, USA) by using digital imaging. Details of the fluorescence imaging and analysis techniques are published (Golovina & Blaustein, 2000). FFP-18 consists of a Ca\(^{2+}\)-sensitive fluorescent Fura group linked via a piperazine group to an acyl chain. The 12-carbon acyl chain is believed to insert into the lipid bilayer while the charged piperazine moiety floats on the surface of the membrane, in a manner similar to a phospholipid head group (Vorndran et al. 1995; Chadborn et al. 2002). FFP-18 is used to monitor rapid, large (up to 4 µM; K\(_d\) for Ca\(^{2+}\) = 0.4 µM) changes in [Ca\(^{2+}\)]\(_{\text{PM}}\) (Davies & Hallett, 1996; Graier et al. 1998). FFP-18 was used here to measure store-operated Ca\(^{2+}\) translocation across the PM.

Astrocytes grown on coverslips were loaded with Fura-2 (30 min) or FFP-18 (2 h) by incubating them in culture medium (20–22°C, 5% CO\(_2\)-95% O\(_2\)) containing 3.3 µM Fura-2-AM or 5 µM FFP-18-AM. Exogenously added FFP-18-AM initially incorporates into the outer leaflet of the PM, but as molecules reorient to face the inner surface by slow ‘flip-flop’ diffusion, cytosolic esterases cleave the ester groups. The Ca\(^{2+}\)-sensitive FFP-18 acid is generated on the inner face and is unable to diffuse back (Davies et al. 1997; Davies & Hallett, 1998).

Figure 1 diagrams the possible distribution of FFP-18 used to image sub-PM microdomains in astrocytes loaded with this dye. Some FFP-18 inserts into the ER membrane mainly facing the PM–ER cytosolic space. The amount of FFP-18 facing the ER lumen appears to be negligible (see Results and Discussion). The FFP-18 signal was
unaffected by application of 2 mM Ni²⁺ (not shown), which quenches the FFP-18 fluorescence on the external face of the PM (Davies & Hallett, 1996). This confirms that the FFP-18 Ca²⁺-sensitive head group was facing into the cell, monitoring cytosolic rather than extracellular [Ca²⁺]. Because the process of 'flip-flop' diffusion is slow compared to diffusion of Fura-2-AM (Davies et al. 1997), time of cell loading with FFP-18-AM is longer than with Fura-2-AM.

After loading with either dye, the coverslips were transferred to a tissue chamber mounted on a microscope stage, where cells were superfused for 15–20 min (35–36°C) with standard physiological salt solution to wash away extracellular dye. The physiological salt solution, PSS, contained (mM): 140 NaCl, 5.9 KCl, 1.2 NaH₂PO₄, 5 NaHCO₃, 1.4 MgCl₂, 1.8 CaCl₂, 11.5 glucose, and 10 Hepes (pH 7.4). Cells were studied for 40–60 min while being continuously superfused with PSS (35°C).

The imaging system was designed around a Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY, USA) optimized for UV transmission. The dye-loaded cells were illuminated with a diffraction grating-based system (Polychrome II, Applied Scientific Instruments, Eugene, OR, USA) (Golovina & Blaustein, 2000). Fluorescence images were recorded by using a General III ultrablue intensified charge-coupled device camera (Stanford Photonics, Palo Alto, CA, USA). Image acquisition and analysis were performed with a MetaFluor/MetaMorph Imaging System (Universal Imaging, West Chester, PA, USA). Cultured astrocytes are particularly well suited for such wide-field fluorescence microscopy because they are very thin (Z axis ≤ 1 µm); thus the ER can be resolved in the X–Y plane. The limit of Z-axis resolution with confocal laser scanning microscopy is ∼0.7 µm (Mrikel et al. 1999). Therefore, confocal microscopy cannot resolve intracellular structures in the Z-axis in cells with a Z-axis dimension ≤ 1 µm.

[Ca²⁺]ᵣ怫 was calculated by determining the ratio of Fura-2 fluorescence excited at 380 and 360 nm as described previously (Golovina & Blaustein, 2000). FFP-18 fluorescence data are presented as 340 nm/380 nm wavelength ratios (510 nm emission).

**Immunofluorescence microscopy**

Astrocytes were immunolabelled, as described by Luther & Bloch (1989). Briefly, cells were fixed in cyclohexylamine–formaldehyde fixative consisting of 0.45% (w/v) formaldehyde, 75 mM cyclohexylamine, 75 mM NaCl, 10 mM EGTA, 10 mM MgCl₂ and 10 mM PIPES. After fixation, the cells were permeabilized in fixative containing 0.5% polyoxyethylene 20 cetyl ether (Brij 58), and were then incubated (4–17 h) in antibody buffer containing antibodies against the various transporters. For surface staining, non-permeabilized cells were blocked with 10% goat serum, and incubated with primary antibody (Antoniotti et al. 2002). The following antibodies were used: rabbit polyclonal anti-TRPC1, anti-TRPC4 (Allomone Laboratories, Israel); rabbit polyclonal anti-TRPC1 (gift of Dr G. Krapivinsky, Harvard Medical School, Boston, MA, USA); rabbit polyclonal anti-α₁D subunit of L-type voltage-gated Ca²⁺ channels (Allomone Laboratories); monoclonal anti-SERCA2 (Affinity BioReagents, Golden, CO, USA); rabbit polyclonal anti-SERCA2b (gift of Dr F. Wuytak, Katholieke University, Leuven, Belgium); rabbit polyclonal anti-PM Ca²⁺-ATPase (gift of Dr E. Carafoli, University of Padova, Italy); rabbit polyclonal anti-IP₃R type 1 (gift of Dr R. J. Wojcikiewicz, State University of New York, Syracuse Health Science Center, Syracuse, NY, USA). FITC-labelled donkey antimouse IgG or Cy3-conjugated donkey antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) were used to visualize the primary antibodies. The fluorescence from the secondary antibody in the absence of primary antibody (positive control) did not exceed 2–3% of the fluorescence in the presence of antisera.

To identify ER in living and fixed astrocytes, cells were treated (5 min) with 200 ng ml⁻¹ 3,3'-dihexyloxacarbocyanine, DiOC₆(3) (DiOC) (Golovina & Blaustein, 2000), or 1 µm ER-Tracker (Molecular Probes, Eugene, OR, USA).

**Immunoprecipitation**

Cultured astrocytes were rinsed, scraped into phosphate-buffered saline (PBS) and subjected to centrifugation for 15 min at 15 000 g and 4°C. The cell pellet was resuspended in ice-cold extraction buffer (1% IGEPAL CA-630 (Sigma), 150 mM NaCl, 2 mM EDTA, 10 mM sodium azide, 20 mM Tris-HCl (pH 7.4) and protease inhibitor mixture tablets (Roche Diagnostics, Germany), followed by 10 passages each through 10- and 25-gauge needles and incubated for 1 h at 4°C. The lysate was centrifuged for 20 min at 27 000 g and 4°C. The supernatant (detergent extract or ‘Homogenate’) was stored at −80°C and used for immunoprecipitation and immunoblotting. The protein concentration was determined with the bicinchoninic acid assay (Bio-Rad), with bovine serum albumin as a standard. Appropriate polyclonal antibodies were incubated with ∼2 × 10⁷ (60 µl) washed magnetic beads (Dynabeads M-450, coated with M-280 sheep antirabbit IgG, Dynal Biotech, Lake Success, NY, USA) overnight at 4°C on a rotator, as described by Lencesova et al. (2004). As negative controls, the coated beads were incubated with rabbit γ-globulin (Jackson ImmunoResearch, West Grove, PA, USA) for polyclonal antibodies raised in rabbits. The beads, with antibody attached, were washed (twice, 200 µl) with PBS. Proteins were immunoprecipitated from 1 mg of
detergent-extracted total protein by incubation for 4 h at 4°C with antibody-bound beads. Following incubation, bead complexes were washed (4 times 400 µl) with PTA (145 mM NaCl, 10 mM NaH2PO4, 10 mM sodium azide, and 0.5% Tween 20, pH 7.0). Immunoprecipitated proteins were then extracted with 50 µl of 2 x Laemmli sample buffer (Bio-Rad) and boiled for 5 min.

**Western immunoblot analysis**

Membrane proteins were separated by 7.5% SDS-PAGE as described by Golovina et al. (2003) and transferred electrophoretically to a nitrocellulose membrane (Amersham BioSciences). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20, incubated overnight at 4°C with appropriate primary antibody. Following washing, membranes were incubated with antirabbit horseradish peroxidase-conjugated IgG for 1 h at room temperature. The immune complexes on the membranes were detected with an enhanced chemiluminescence detection system (ECL Plus; Amersham BioSciences).

**Antisense oligonucleotides**

Next Generation™ antisense oligonucleotides (AS-oligos) were synthesized by Sequitur, Inc. (Natick, MA, USA). AS-oligos were directed at the TRPC1 coding sequences (AUAGUCACCCUUGUCGACCGCAGC). Oligos with the same base composition, but with a scrambled sequence (CCACCUGAGUUCAACGUGUCC) controlled for non-specific or toxic effects of the oligos. The oligonucleotides were transfected into cells by Lipid 2012-G (Seqitur). Astrocytes were divided into three groups: (i) the antisense group was exposed to the AS-oligos; (ii) one control group was exposed to the scrambled (nonsense, NS) oligos; and (iii) the second control group was grown without oligos. For each treatment, the cells were first rinsed with Opti-MEM, after which the oligos were added to the cells. Following 16–24 h incubation, the medium was aspirated and replaced with growth medium without oligos for 48–60 h before Ca2+ measurement or immunocytochemistry was performed.

**Statistical analysis**

The numerical data presented in Results are the means ± s.e.m. from n single cells (one value per cell). Immunofluorescence labelling experiments were repeated at least four times. The number of different animals and different litters are also presented, where appropriate. Data from four to five litters were obtained for most protocols and were consistent from litter to litter. Statistical significance was determined using Student’s t test and ANOVA.

**Results**

**Store-operated Ca2+ influx colocalizes to the ER**

When astrocytes are loaded with FFP-18, the hydrophobic analogue of Fura-2 (Etter et al. 1994; Davies & Hallett, 1996), much of the fluorescence signal comes from the inner surface of the PM and reflects [Ca2+]i in that vicinity, i.e. [Ca2+]SPM (Fig. 2). FFP-18 also inserts into the ER membrane, mainly facing the PM–ER cytosolic space (Fig. 1). There appears to be little or no contamination of the sub-PM signal with a signal from the lumen of the ER because the resting FFP-18 ratio image is relatively uniform (Fig. 2Da). Identification of the ER was confirmed at the end of each experiment by staining with DiOC (Fig. 2C and F).

Figure 2B shows the time course of changes in the FFP-18 ratio in an area overlying the ER (red) and in an ER-free region (blue), in response to the application of cyclopiazonic acid (CPA, an ER Ca2+ pump blocker) and caffeine (CAF). First, the Ca2+ stores were depleted in the absence of extracellular Ca2+. Then, following store depletion, when changes in the [Ca2+]SPM signal were no longer observed (~15 min), Ca2+ was added back and the rise in [Ca2+]SPM due to SOCE was measured. To eliminate the contribution of Ca2+-induced Ca2+ release (CICR) to the SOCE-evoked rise in [Ca2+]SPM, the solutions contained 1 µM ryanodine (RY). RY at this concentration locks the CICR channels in an open, low-conductance state (Smith et al. 1988); this promotes depletion of the CAF/RY-sensitive store by CAF (Golovina & Blaustein, 2000). The rise in [Ca2+]SPM due to SOCE starts in the PM regions overlying the ER (Fig. 2De, E and G); it is followed with a delay by Ca2+ increases in ER-free regions, as a result of Ca2+ diffusion. Note that the sites of SOCE (Figs 2E and G) colocalize with the sites of CPA + CAF-induced ER Ca2+ release (Fig. 2E), indicating their close association. The rates of Ca2+ diffusion from the sites of Ca2+ release and SOCE are comparable (5.5 ± 0.8 and 6.1 ± 1.2 µm s−1, respectively; n = 12 cells). These rates are, however, lower than velocity of agonist (ATP)-induced Ca2+ waves in cultured astrocytes (17 ± 6 µm s−1; n = 58 cells), comparable to published data for spread of Ca2+ waves (19 ± 9 µm s−1; Cornell-Bell et al. 1990). This 3-fold difference in velocity may be explained by regenerative Ca2+ release along the length of the cell (Yagodin et al. 1995). SOCE can be heterogeneous. For example, in the area overlying the element of ER indicated by the lower arrowhead, the SOCE signal was larger than in the adjacent area (upper arrowhead). This is likely to be the consequence of a high density of SOC expression in these PM
Visualization of store-operated calcium entry

Microdomains and restricted Ca$^{2+}$ diffusion in the tiny volume of cytosol located between the ‘junctional’ ER and the PM (Blaustein & Golovina, 2001). Modulation of PM Ca$^{2+}$-ATPase activity by local Ca$^{2+}$ microdomains near SOCs (Bautista & Lewis, 2004) and/or regional differences in Ca$^{2+}$ binding proteins might also contribute to local gradients of [Ca$^{2+}$]$_{SPM}$ in the regions overlying the ER (Fig. 2D).

The sites of SOCE signal initiation were compared with localization of Ca$^{2+}$ influx through L-type voltage-gated Ca$^{2+}$ channels (‘contrast control’; Fig. 3). In marked contrast to the SOCE signal, 50 mM K$^+$-induced Ca$^{2+}$ influx, which is blocked by dihydropyridines (not shown), and thus mediated by L-type Ca$^{2+}$ channels, is uniform in all regions of the PM (Fig. 3B–D). To increase voltage-activated Ca$^{2+}$ influx, high K$^+$ was applied with a Ca$^{2+}$ channel agonist, BayK 8644 (10 µM) (MacVicar et al. 1991). The rate of high K$^+$-induced rise in [Ca$^{2+}$]$_{SPM}$ is 4- to 5-fold higher (Fig. 3B and C inset) than that due to SOCE (Fig. 2B inset, and G). This is likely the consequence of a higher density and/or larger conductance of L-type Ca$^{2+}$ channels than SOCs (Parekh & Penner, 1997).

Inhibition of TRPC1 expression with antisense oligos reduces SOCE

Molecular identification of SOCs in astrocytes is essential for studying functional and spatial expression of SOCE. Figure 4 shows that mouse cortical astrocytes express TRPC1, which is an obligatory component of endogenous SOCs in a variety of cell types (Liu et al. 2000; Harteneck et al. 2000; Xu & Beech, 2001).

To determine whether TRPC1 is involved in SOCE in astrocytes, an antisense strategy was employed. Selective

![Figure 2. SOCE-induced local [Ca$^{2+}$]$_{SPM}$ signal in an astrocyte](image)

A, FFP-18 ($F_{360}$) image of a portion of cell. To better visualize the ER, the image contrast was increased so that the ER appears to contain much more dye than is actually the case. N, nucleus. Scale bar = 25 µm. B, time course of ratio ($F_{340}/F_{380}$) signal in regions outlined in A (red and blue); times of treatment with CPA (10 µM) + CAF (10 mM) and Ca$^{2+}$-free solution are indicated. RY (1 µM) was applied 10 min before the traces shown and was maintained throughout the experiment. Inset shows the early rising phase of the $F_{340}/F_{380}$ ratio after Ca$^{2+}$ restoration. C, DiOC image of the boxed portion of A. Arrowheads point to ER (including mitochondria). Most mitochondria, which are brightly stained by DiOC, appeared to lie on the ER. D, ratio ($F_{340}/F_{380}$) images (a–j) captured at the times indicated in B. E, linescan of CPA + CAF-induced changes in ratio ($F_{340}/F_{380}$), shown in B, along the white dotted line in C and F. The time scale is variable. G, linescan with high time scale resolution (from the boxed portion in E). Scale bar = 10 s. Comparable results were obtained in 12 other cells.
inhibition of TRPC1 protein expression, using AS-oligos targeted to the TRPC1 gene (Fig. 4A and B), significantly attenuated the SOC-mediated rise of $[\text{Ca}^{2+}]_{\text{cyt}}$, measured with Fura-2 (311 ± 18 versus 1090 ± 48 nm in cells treated with NS-oligos) (Fig. 4C and D). The cells in all three groups retained normal morphology, but astrocyte proliferation was inhibited in the AS-oligos treated group (Fig. 4F). TRPC1 knockout cells were distinguished from non-transfected cells by staining with anti-TRPC1 antibody (Fig. 4Eb) immediately after physiological experiments with Fura-2. The cells were then also labelled with the nucleic acid stain 4′,6′-diamidino-2-phenylindole (DAPI, 5 µm) to estimate the total cell number in the field (Fig. 4Ec). Figure 4 shows that 6 of the 8 cells treated with AS-oligos did not express detectable TRPC1 (Fig. 4Ea and b) and exhibited significantly attenuated SOCE (Fig. 4C). Some nuclei also appear to be slightly fluorescent (Fig. 4Eb) due to non-specific labelling by the secondary antibody (see Methods). All untreated (control) cells expressed TRPC1 (not shown). These results strongly suggest that TRPC1 is an essential component of the endogenous SOCs that mediate SOCE in mouse cortical astrocytes. Moreover, the results support the view that TRPC1 gene expression regulates astrocyte proliferation by modulating SOCE activity (Golovina et al. 2001).

**TRPC proteins localize to PM microdomains adjacent to underlying ER**

High resolution immunocytochemistry was used to elucidate the relationship between the sites of SOCE signal initiation (Fig. 2) and the specific location of TRPC proteins in the PM. Figure 5 shows the distribution of endogenous TRPC proteins in intact (non-transfected) astrocytes. The low magnification image demonstrates that TRPC1 label is distributed in a distinct reticular pattern (Fig. 5A) that parallels the organization of the underlying ER-Tracker-stained ER (Fig. 5B). This reticular pattern indicates that clusters of TRPC1 in the PM are organized around the underlying ER.

High power images of a portion of another astrocyte show that the reticulate TRPC1 labelling pattern (Fig. 5Da) is remarkably similar to the pattern observed with antibodies directed against the ER $\text{Ca}^{2+}$ pump, SERCA2 (Fig. 5Db) or with ER-Tracker (Fig. 5Dc). The confinement of TRPC1 to PM microdomains that overlie ER in astrocytes is consistent with the proposed role of this protein as an essential component of SOC. The identical TRPC1 and SERCA distribution is likely to have functional significance, as SOCE is a major pathway involved in refilling ER $\text{Ca}^{2+}$ stores: SOCE-regulated ER stores communicate with the PM to modulate $\text{Ca}^{2+}$ entry as a function of ER $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_{\text{ER}}$) (Hofer et al. 1998). Notably, reactivity was not detected in the PM in the absence of the primary antibody (not shown) or when anti-TRPC1 antibody was first incubated with the peptide (Fig. 5C).

TRPC channels are assembled as homo- or hetero-TRPC tetramers (Hoffman et al. 2002). In particular, TRPC1 has the unique ability to form channel complexes together with TRPC4 and TRPC5 (Hoffman et al. 2002). As shown in Fig. 5E, TRPC4 protein also localizes to PM microdomains adjacent to underlying ER. To confirm that the reticulate pattern of TRPC labelling is not an

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**Figure 3. High K⁺-induced $[\text{Ca}^{2+}]_{\text{PM}}$ signal in an astrocyte**

A, FFP-18 (F360) image of a small portion of cell; scale bar = 25 µm. B, linescan of high K⁺-induced changes in ratio along the white dotted line in A. C, time courses of ratio ($F_{340}/F_{380}$) signal in regions outlined in A (red and blue); time of treatment with 50 mM KCl is indicated. Inset shows the early rising phase of the $F_{340}/F_{380}$ ratio. D, ratio ($F_{340}/F_{380}$) images captured at the times indicated in C inset. Comparable results were obtained in eight other cells.
artifact, its distribution is compared with that of the PM Ca\textsuperscript{2+} pump, which is distributed uniformly over the cell surface (Juhaszova et al. 1997). TRPC labelling is also compared with distribution of L-type voltage-gated Ca\textsuperscript{2+} channels (\(\alpha_{1D}\) subunit), which are expressed in astrocytes (Latour et al. 2003). In contrast to the TRPC proteins, PM Ca\textsuperscript{2+} pump and L-type Ca\textsuperscript{2+} channel labelling are clearly different from the reticular pattern of SERCA2 (Fig. 5Fa and b) or ER labelling (Fig. 5Ga and b).

TRPC1 is a PM protein spanning the membrane with an extracellular domain (Xu & Beech, 2001). To confirm the PM localization of endogenous TRPC protein in astrocytes immunocytochemical experiments were repeated on non-permeabilized cells by using TRPC1 antibody targeted to peptide predicted to contribute to the outer vestibule of the pore, between the fifth and the sixth transmembrane segments (Antoniotti et al. 2002). Figure 5H shows a reticular pattern of TRPC1 PM staining, but no SERCA2 (i.e. ER) labelling in non-permeabilized triple-stained cell. Background fluorescence (Fig. 5Hb) is non-specific from the secondary antibody and did not exceed 2–3% of the fluorescence of permeabilized cells in the presence of SERCA2b antibody (Fig. 5Db). ER-Tracker (Fig. 5Hc) which also stains non-permeabilized cells, has a distribution similar to TRPC1 (Fig. 5Ha). These data are consistent with the FFP-18 results demonstrating a distinct reticular pattern of distribution of SOCE signal (Fig. 2).

The colocalization of TRPC-encoded SOCs with the ER at PM–ER junctions in astrocytes suggests that there is a special structural relationship between TRPCs and elements of the ER. To confirm this, immunoprecipitates (IPs) of endogenously expressed TRPC1 were prepared from detergent-solubilized extracts of mouse astrocyte membranes. Immunoblots were performed on these IPs to determine whether TRPC1 forms a complex with ER Ca\textsuperscript{2+}.
transporters and receptors. Co-immunoprecipitation data revealed that TRPC1 in mouse cortical astrocytes, indeed, interacts not only with IP₃R type 1, but also with SERCA2b (Fig. 6A). For controls, the Dynabeads were coated with non-specific rabbit γ-globulin (γ-Gl) for polyclonal antibodies. No TRPC1 bound to these beads. The data were confirmed by complementary (converse) experiments: IP with an antibody rose against SERCA2b or IP₃R-1 coimmunoprecipitated TRPC1 (Fig. 6B). TRPC1-reactive band was not found in SERCA2b IP when anti-TRPC1 antibody was preincubated with antigen peptide (not shown). This demonstrates that the coimmunoprecipitated immunoreactive band in the SERCA2b IP is TRPC1. The results indicate that there is a physical association between TRPC channels and ER Ca²⁺ stores.

**Discussion**

The experiments described here demonstrate that direct visualization of localized SOCE signals is possible by using wide-field imaging with the near-membrane Ca²⁺ indicator, FFP-18. The data indicate that SOCE in primary cultured mouse cortical astrocytes occurs at PM-ER junctions. This conclusion is confirmed by immunocytochemical observations and by coimmunoprecipitation data showing that PM microdomains adjacent to the underlying ER contain TRPC channel proteins which are components of SOCs.

**Association between the PM and ER**

SOCE is a mechanism that links [Ca²⁺]ER to PM Ca²⁺ permeability and serves as an important pathway to refill

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**Figure 5. Immunofluorescent localization of TRPC proteins in astrocytes**

A and B, low magnification images of cells crossreacted with anti-TRPC1 antibody (A), ER-Tracker was later used to stain the ER (B). C, fluorescence detected when the primary antibody was preincubated with TRPC1 peptide. D, high magnification images of a portion of an astrocyte triple labelled with anti-TRPC1 antibody (a), anti-SERCA-2 antibody (b), and ER-Tracker (c). All three labels show similar reticular distributions. E, images of a portion of another astrocyte double labelled with anti-TRPC4 antibodies (a), and ER-Tracker (b). F, high power images of a portion of astrocyte double labelled with anti-PM Ca²⁺-ATPase (a) and anti-SERCA-2 antibodies (b). G, images of a portion of astrocyte double labelled with anti-L-type Ca²⁺ channel antibody (a) and ER-Tracker (b). H, images of a portion of a non-permeabilized astrocyte double labelled with anti-TRPC1 antibody (a), anti-SERCA-2 antibody (b), and ER-Tracker (c). Scale bars = 25 μm (A and C), 5 μm (Da, Ea and Fa), and 10 μm (Ga and Ha). Similar results were obtained in 18 cells (A and B), 53 cells (D), 37 cells (E), 14 cells (F), 11 cells (G), and 15 cells (H) from 16 fetuses (4 litters).
intracellular Ca\(^{2+}\) stores. Therefore one may conjecture that specificity of such intimate functional interaction between ER Ca\(^{2+}\) stores and PM SOCs is determined by their spatial proximity (Berridge, 1995; Peterson & Berridge, 1996; Jaconi et al. 1997; Putney, 1999). Indeed, in many cell types, the peripheral ER membrane frequently comes within 8–20 nm of the PM, with which it appears to form ‘junctions’ with periodic structures spanning the gap (Henkart et al. 1976; Watanabe & Burnstock, 1976; Somlyo & Franzini-Armstrong, 1985). In neurons, PM–junctional ER units appear structurally similar to the PM–sarcoplasmic reticulum (SR) junctions in the triads and diads of skeletal and cardiac muscle (Henkart et al. 1976; Blaustein & Golovina, 2001). Van Breemen et al. (1995) suggest that there is a second space between the PM and SR that is narrow and restricted, but not ‘junctional’. Diffusion of ions from such PM–superficial ER compartments to ‘bulk’ cytosol must be markedly restricted (Van Breemen et al. 1995; Delmas & Brown, 2002). Therefore Ca\(^{2+}\) entering the cells is directly accumulated by the peripheral ER without altering bulk \([\text{Ca}^{2+}]_{\text{cyt}}\) (Van Breemen et al. 1995; Blaustein & Golovina, 2001; Flemming et al. 2002). This may explain why Ca\(^{2+}\) influx fails to induce contraction in some smooth muscle when SR Ca\(^{2+}\) stores are depleted by SERCA inhibitors (Flemming et al. 2002). In cells undergoing continuous Ca\(^{2+}\) oscillations, the entry of Ca\(^{2+}\) to replenish repeatedly releasing Ca\(^{2+}\) stores is critical (Venkatachalam et al. 2002).

SOCE occurs at PM–ER junctions

Close association between some ER Ca\(^{2+}\) release sites and SOCE has been reported in Xenopus oocytes. For instance, agonist-evoked ER Ca\(^{2+}\) release in restricted regions stimulates Ca\(^{2+}\) entry-activated chloride currents only in those same regions (Peterson & Berridge, 1996). Also, following centrifugation of Xenopus oocytes to redistribute their organelles, thapsigargin-induced Ca\(^{2+}\) release localizes to the ER layer and Ca\(^{2+}\) enters in adjacent PM regions (Jaconi et al. 1997). The sites of SOCE, however, have not been visualized in astrocytes or any other type of cells with intact intracellular morphology.

Localization of Ca\(^{2+}\) entry in the vicinity of SOCs requires high spatial resolution. The distance between the PM and the junctional ER (≈20 nm) is less than the optical resolution of light microscopes. Therefore, these tiny sub-PM compartments have not been imaged by confocal, deconvolution or two-photon microscopy, all of which have Z-axis resolution ≤ 0.7 μm (Juhaszova & Blaustein, 1997; Mriel et al. 1999). Measurement of \([\text{Ca}^{2+}]_{\text{SPM}}\) with total internal reflection fluorescence (TIRF) is also limited because Z-axis resolution is about one-fifth the effective thickness of confocal or two-photon fluorescence, i.e. ≈ 150–200 nm (Axelrod, 2001). Thus, measurements of Ca\(^{2+}\) in the restricted space between the PM and junctional ER (≈20 nm) also cannot be resolved by TIRF. Therefore alternative methods are required. To monitor \([\text{Ca}^{2+}]_{\text{SPM}}\) membrane-tethered Ca\(^{2+}\) indicators, such as FFP-18, have been developed (Etter et al. 1994; Vorndran et al. 1995; Lloyd et al. 1995). Several reports demonstrate that this indicator does, indeed, monitor changes in \([\text{Ca}^{2+}]_{\text{SPM}}\) rather than the bulk \([\text{Ca}^{2+}]_{\text{cyt}}\) (Davies et al. 1997; Paltauf-Dobrzynska et al. 1998; Chadborn et al. 2002).

This study reveals that SOCE starts at PM regions overlying the ER. The Ca\(^{2+}\) then spreads relatively slowly to nearby cytosol as long as SERCA is inhibited (Fig. 2).

**Figure 6. Coimmunoprecipitation of endogenous TRPC1 with SERCA2b and IP3R-1 in mouse cortical astrocytes**

A, TRPC1 immunoprecipitate (IP) was generated and probed with anti-SERCA2b and anti-IP3R type 1 antibodies. Control beads were prepared with rabbit γ-globulin (γ-G1). Gels were loaded with homogenate (Homog, 1st lane; 2 μg protein for SERCA2b and 10 μg protein for IP3R-1), IP pellet (IP P, 2nd lane; 10 μl of IP protein solution for all co-IPs); post-IP supernatant (IP S, 3rd lane, same volume as 1st lane) and pellet eluted from γ-globulin-IP beads (Con P, 4th lane). B, SERCA2b and IP3R-1 IPs probed with TRPC1 antibody. Reactivity to anti-TRPC1 was blocked by preincubating the antibody with the peptide (not shown). Molecular masses are indicated in kDa.

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FFP-18 may also insert into the ER membrane, but mainly facing the PM–ER cytosolic space (Fig. 1). Localization of dye molecules in the ER membrane facing the ER lumen is negligible, as confirmed by the relatively uniform resting FFP-18 ratio image (Fig. 2Da). (FFP-18 in the ER membrane facing the lumen should be saturated with Ca\textsuperscript{2+} because [Ca\textsuperscript{2+}]\textsubscript{ER} > 150 μM (Golovina & Blaustein, 1997), this is about 375 times larger than the FFP-18 K\textsubscript{d} (0.4 μM). This would give rise to a non-uniform resting FFP-18 ratio image, which was not observed. Furthermore, the expected decline in [Ca\textsuperscript{2+}]\textsubscript{ER} during store depletion cannot be confused with the rise in [Ca\textsuperscript{2+}]\textsubscript{SPM} caused by SOCE because the [Ca\textsuperscript{2+}] signals from the ER and sub-PM space should move in opposite directions.) Furthermore, when the ER Ca\textsuperscript{2+} stores are unloaded for 15 min in Ca\textsuperscript{2+}-free media, the FFP-18 ratio (F\textsubscript{340}/F\textsubscript{380}) first increases, indicating a rise in [Ca\textsuperscript{2+}]\textsubscript{SPM}, and then returns to the same initial level (Fig. 2Da and b), i.e. the FFP-18 signal does not reflect the expected changes in ER luminal Ca\textsuperscript{2+}. Moreover, external Ca\textsuperscript{2+} was restored and SOCE was activated only when localized [Ca\textsuperscript{2+}]\textsubscript{SPM} changes following store depletion were no longer observed (Figs 2B and E). Particularly noteworthy is the fact that the sites of SOCE signal initiation (Fig. 2G) are remarkably different from the sites of Ca\textsuperscript{2+} influx through L-type voltage-gated Ca\textsuperscript{2+} channels, which are ubiquitously distributed in all regions of the PM (Fig. 3B). This approach permits the visualization of localized [Ca\textsuperscript{2+}]\textsubscript{SPM} changes even if the precise magnitude of SOCE-induced changes in [Ca\textsuperscript{2+}]\textsubscript{SPM} is more difficult to resolve. The data presented here indicate that SOCE has preferred access to the ER.

PM–ER compartments might include mitochondria (Dahl et al. 1965; Mali et al. 2003), which are able to buffer Ca\textsuperscript{2+} entering through both SOCs and voltage-gated Ca\textsuperscript{2+} channels (Parekh, 2003). Mitochondria can generate sustained sub-PM microdomains of low [Ca\textsuperscript{2+}] and modulate SOCE by controlling its magnitude and duration (Malli et al. 2003; Parekh, 2003): this does not, however, alter the conclusion that SOCs are confined to PM–ER junction compartments. Mitochondrial buffering might contribute to the heterogeneity of localized SOCE signals in the regions overlying the ER (Fig. 2D). Further studies are required to determine the impact of mitochondrial Ca\textsuperscript{2+} signalling on localized SOCE in cortical astrocytes.

**Endogenous TRPC1 is involved in SOCE in astrocytes**

Mammalian SOCs are believed to consist of TRPC protein tetramers (Harteneck et al. 2000; Xu & Beech, 2001). The presence of TRPC proteins in astrocytes has previously been demonstrated (Pizzo et al. 2001; Grimaldi et al. 2003) although their involvement in SOCE is not clear. For instance, Pizzo and colleagues compared TRPC expression in rat cortical astrocytes and RBL-2H3 cells and did not find a simple correlation between the level of TRPC expression and the magnitude of SOCE. It is commonly believed that within the TRPC family TRPC1 is an obligatory component of endogenous SOCs in a variety of cell types (Harteneck et al. 2000). The present study, using antisense oligos, demonstrates that TRPC1 is also involved in SOCE in mouse cortical astrocytes. Cell transfection with antisense oligos targeted to the TRPC1 gene reduced, by ~70%, both expression of TRPC1 protein (Fig. 4A and B) and SOCE (Fig. 4D).

**TRPC proteins localize to the PM microdomains adjacent to the underlying ER**

The distribution of the TRPC proteins in the PM of astrocytes has not been previously described. Low-resolution immunocytochemical studies in other cells, however, indicate that endogenous TRPCs are not distributed uniformly. For example, in primary cultured human myometrial smooth muscle cells, TRPC1, TRPC3, TRPC4 and TRPC6 proteins appear to be distributed in a reticular fashion (Dalrymple et al. 2002). In a line of native cultured bovine aortic endothelial cells (BAE-1 cells), the TRPC1 labelling is also punctate (Antoniotti et al. 2002; Fig. 3A), suggesting that this protein is distributed in an organized manner in the PM. In the present study clear reticular patterns of PM distribution of TRPC proteins were demonstrated by using high spatial resolution immunocytochemistry on permeabilized as well as on non-permeabilized astrocytes. There is striking similarity in the distribution of TRPC proteins and ER labelling with SERCA2 or ER-tracker in both permeabilized (Fig. 5D) and non-permeabilized astrocytes (Fig. 5Ha and c); in contrast, no immunofluorescent SERCA2 labelling (i.e. ER) is observed in non-permeabilized cells (Fig. 5Hb). This observation demonstrates that TRPC proteins are present on the cell surface. The data confirm previous reports demonstrating PM localization of TRPC1 protein in native non-permeabilized vascular smooth muscle cells (Xu & Beech, 2001) and in endothelial cells (Antoniotti et al. 2002). The reticular pattern of PM localization of TRPC-encoded SOCs is consistent with the close proximity of the SOCE signals to the ER. This differs from the uniform distribution of L-type voltage-gated Ca\textsuperscript{2+} channels and high K\textsuperscript{+}-induced Ca\textsuperscript{2+} influx.

**TRPC1 forms a Ca\textsuperscript{2+} signalling complex with ER Ca\textsuperscript{2+} transporters**

Coimmunoprecipitation data indicate that, when TRPC proteins are transfected into heterologous cells, they interact with endogenous IP\textsubscript{3}Rs (Boulay et al. 1999;
Kiselyov et al. 2000; Tang et al. 2001) or with RY receptors (Kiselyov et al. 2000). The present study demonstrates that endogenous TRPC1 in primary cultured astrocytes coimmunoprecipitates not only with IP$_3$Rs but also with SERCA2b (Fig. 6); this is consistent with the immunocytochemical data showing the structural relationship between the TRPC1 and SERCA2 (Fig. 5D). The data support the idea that PM microdomains containing TRPC channels form Ca$^{2+}$ signalling complexes with adjacent ER Ca$^{2+}$ transporters in astrocytes. PM microdomains containing TRPC channels might be linked to the underlying junctional ER by adaptor/scaffolding proteins (Yuan et al. 2003). In a recent study Treves et al. (2004) demonstrated that PM–ER compartments are stabilized by ‘junctate’, an integral Ca$^{2+}$ binding protein of ER membrane, that forms a supramolecular complex with the IP$_3$R and the TRPC3 and modulates Ca$^{2+}$ entry through receptor- and store-activated channels in T3-HEK293 cells.

In summary, this report directly demonstrates that TRPC-encoded SOCs in astrocytes, and the SOCE they mediate, are situated very close to the sub-PM ER. Such specialized distribution of PM SOCs, relative to the ER, facilitates functional, and possibly structural coupling of SOCs to the ER Ca$^{2+}$ stores. In this way, the Ca$^{2+}$ content of the ER can be regulated independently of changes in bulk cytoplasmic [Ca$^{2+}$].

Numerous observations indicate that PM microdomains that overlie the closely apposed junctional ER compartments are functionally specialized because of their unique complements of ion transporters (Blaustein & Golovina, 2001; Lencesova et al. 2004). In astrocytes, for instance, these PM microdomains contain clusters of isoform-specific Na$^+$ pumps (α2) and Na$^+$–Ca$^{2+}$ exchangers (Juhaszova et al. 1997). As shown here, these PM microdomains also contain TRPC-encoded SOCs. Thus, the PM–junctional ER complexes function as integrated units that regulate the initiation and spread of Ca$^{2+}$ signals in astrocytes.

References


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