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The cytoskeleton plays a modulatory role in the association between STIM1 and the Ca^{2+} channel subunits Orai1 and TRPC1

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ABSTRACT

Store-operated Ca^{2+} entry (SOCE) is a major pathway for Ca^{2+} influx in non-excitable cells. Recent studies favour a conformational coupling mechanism between the endoplasmic reticulum (ER) Ca^{2+} sensor STIM1 and Ca^{2+} permeable channels in the plasma membrane to explain SOCE. Previous studies have reported a role for the cytoskeleton modulating the activation of SOCE; therefore, here we have investigated whether the interaction between STIM1 and the Ca^{2+} permeable channels is modulated by the actin or microtubular network. In HEK-293 cells, treatment with the microtubular disrupter colchicine enhanced both the activation of SOCE and the association between STIM1 and Orai1 or TRPC1 induced by thapsigargin (TG). Conversely, stabilization of the microtubules by paclitaxel attenuated TG-evoked activation of SOCE and the interaction between STIM1 and the Ca^{2+} channels Orai1 and TRPC1, altogether suggesting that the microtubules act as a negative regulator of SOCE. Stabilization of the cortical actin filament layer results in inhibition of TG-evoked both association between STIM1, Orai1 and TRPC1 and SOCE. Interestingly, disruption of the actin filament network by cytochalasin D did not significantly modify TG-evoked association between STIM1 and Orai1 or TRPC1 but enhanced TG-stimulated SOCE. Finally, inhibition of calmodulin by calmidazolium enhances TG-evoked SOCE and disruption of the actin cytoskeleton results in inhibition of TG-evoked association of calmodulin with Orai1 and TRPC1. Thus, we demonstrate that the cytoskeleton plays an essential role in the regulation of SOCE through the modulation of the interaction between their main molecular components.

1. Introduction

Store-operated Ca^{2+} entry (SOCE) is a major mechanism for Ca^{2+} influx into cells and plays an important role in a wide variety of cellular processes [1]. The endoplasmic reticulum (ER) Ca^{2+} sensor STIM1, as well as a number of Ca^{2+}-permeable plasma membrane channels, including Orai1 and certain members of the TRPC subfamily, are key players in SOCE. STIM1 is a single-pass membrane protein that is mainly located in the ER membrane and shows different functional domains, including the luminal EF hands, that allow STIM1 to sense the intraluminal Ca^{2+} concentration [2,3], the Orai1 interaction domain (SOAR/CAD) [4–6], the CRAC modulatory domain (CMD) [7] and the STIM1 homomerization domain (SHD) [8], among others. When Ca^{2+} stores are full STIM1 remains in an inactive state through an intramolecular shielding of the SOAR/CAD domain that prevents constitutive SOCE [8]. However, discharge of the ER results in dissociation of Ca^{2+} from the EF-hand domains, thus resulting in the activation of the store-operated channels (SOCs) Orai1 and TRPCs. The coupling between STIM1 and Orai1 involves the interaction of SOAR domain positive charges with the acidic domain within the Orai1 C-terminal domain in order to activate the channel [9,10]. On the other hand, STIM1 has been shown to interact and gate TRPC members, such as TRPC1, through electrostatic interaction between STIM1 (K684,K685) and TRPC1 (D639,D640) [11].

Recent studies in HEK-293 cells have reported that, while oligomerization of STIM1 is independent on the microtubular network, the distribution of STIM1 in the ER requires the integrity of the microtubules, which facilitate STIM1 movements [12]; thus providing evidence for a role of the cytoskeleton in the regulation of SOCE in these cellular model. A number of studies have investigated the regulation of SOCE by the cytoskeleton, including the actin microfilaments and the microtubules, with different results depending on the cell type. In NIH 3T3 fibroblasts and smooth muscle DT40 cells inhibition of actin polymerization with cytochalasin or depolymerization of the microtubular network with nocodazole does not significantly alter SOCE [13–16], thus suggesting that in these cells the cytoskeleton is not essential for the activation of SOCE. In contrast, in human platelets, which have a more evenly distributed cytoskeleton, impairment of actin

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filaments and microtubules remodelling either by inhibition of polymerization or by stabilization results in inhibition of SOCE [17–19] and a similar functional role has been described for the actin cytoskeleton in endothelial cells [20] and pancreatic acinar cells [21]. In human platelets we have more recently reported that store depletion results in an initial decrease in the actin filament content, probably due to disorganization of the cortical actin network to facilitate the interaction between proteins in the ER and plasma membrane, followed by an increase in actin polymerization necessary for the intracellular trafficking of portions of the ER towards the plasma membrane [22]. Therefore, we have explored the functional relevance of the cytoskeleton in the association between STIM1 and the SOC components Orai1 and TRPC1 upon depletion of the intracellular Ca^{2+} stores in HEK-293 cells endogenously expressing these proteins.

2. Materials and methods

2.1. Materials

Fura-2/AM and Oregon Green 488 paclitaxel were from Invitrogen (Madrid, Spain). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), Fluorescein isothiocyanate (FITC) conjugated phalloidin, Tris, triton X-100, phenyl methyl sulfonyl fluoride, leupeptin, benzamidin, deoxycholate, thapsigargin (TG), probenecid, Nonidet P-40, formaldehyde, calmidazolium, colchicine and paclitaxel (Taxol) were from Sigma (Madrid, Spain). Cytochalasin D (Cyt D) and jasplakinolide were from Calbiochem (Madrid, Spain). Dulbecco's modified Eagle's medium and heat-inactivated fetal bovine serum were from Sanex (Badajoz, Spain). Mouse anti-STIM1 antibody (25-139) was from BD Transduction Laboratories (Frankin Lakes, NJ, USA). Rabbit anti-hTRPC1 polyclonal antibody (557-571) was obtained from Alomone Laboratories (Jerusalem, Israel). Rabbit anti-Orai1 polyclonal antibody N terminal was from ProSci Inc (Derio, Bizkaia, Spain). Mouse anti-calmodulin antibody was from Abcam (Cambridge, UK). Donkey anti-rabbit IgG horseradish peroxidase-conjugated, sheep anti-mouse IgG horseradish peroxidase-conjugated, blotting paper and photographic films were from GE Healthcare (Madrid, Spain). Protein A-agarose was from Upstate Biotechnology Inc (Waltham, MA, USA). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). Hyperfilm ECL was from Amersham (Buckinghamshire, UK). All other reagents were of analytical grade.

2.2. Cell culture

Human embryonic kidney 293 (HEK-293) cells were obtained from the American Type Culture Collection (Barcelona, Spain) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, in a 37 °C incubator with 5% CO_2. At the time of the experiments cells were suspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, pH 7.45. When a Ca^{2+}-free medium was required 1.2 mM EGTA was added.

2.3. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_c$)

HEK-293 cells were suspended and loaded with fura-2 by incubation with 2 μM fura-2/AM and 2.5 mM probenecid for 30 min at 37 °C. Fluorescence was recorded from 2 mL aliquots of magnetically stirred cellular suspension (2×10^6 cells/mL) at 37 °C using a Cary Eclipse spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated in terms of $[Ca^{2+}]_c$ [23].

Ca^{2+} release by TG was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after the addition of the agonist. Ca^{2+} entry into TG-treated cells upon addition of Ca^{2+} to the extracellular medium was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ ($\int\Delta[Ca^{2+}]_c\,dt$), normalized taking a sample every second, and expressed as nM s [24–26]. Ca^{2+} entry was corrected by subtraction of the $[Ca^{2+}]_c$ elevation due to leakage of the indicator to eliminate interferences due to extracellular fura-2.

2.4. Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [27]. Briefly, cell suspension aliquots (500 μL; 2×10^6 cells/mL) were treated as described and lysed with lysis buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% Triton X-100, 2 mM phenyl methyl sulfonyl fluoride, 100 μg/mL leupeptin and 10 mM benzamidine. Samples were immunoprecipitated by simultaneous incubation with 2 μg of anti-STIM1 antibody and protein A-agarose overnight at 4 °C on a rocking platform. Proteins were separated by 10% SDS-PAGE and electrophoretically transferred, for 2 h at 0.8 mA/cm^2, in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs., U.K.) onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of Orai1, hTRPC1, calmodulin and STIM1 was achieved using the anti-Orai1, anti-calmodulin or anti-STIM1 antibodies diluted 1:1000 in TBST for 2 h or the anti-hTRPC1 antibody diluted 1:200 in TBST for 2 h. To detect the primary antibody, blots were incubated for 1 h with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:10,000 in TBST and then exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films. The density of bands on the film was measured using ImageJ (Windows version; National Institutes of Health) [28].

To assess the specificity of the bands we performed primary controls and primary-free controls, where whole cell lysates (2×10^6 cells/mL) were subjected to 10% SDS-PAGE and proteins were electrophoretically transferred onto membranes for incubation with primary and subsequently secondary antibody (primary control) or incubation solely with secondary antibody (primary-free control).

2.5. Measurement of F-actin content

The F-actin content was determined according to a previously published procedure [22]. Briefly, samples of cell suspensions (200 μL; 2×10^6 cells/mL) were transferred to 200 mL of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS; composition, in mM: NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.3) for 10 min. Fixed cells were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved in PBS. Cells were then incubated for 30 min with 1 μM FITC-labelled phalloidin in PBS supplemented with 0.5% (w/v) BSA. Cells were collected by centrifugation and resuspended in PBS. Cell staining was measured using a Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). Samples were excited at 496 nm, and emission was at 516 nm.

2.6. Measurement of microtubular content

The microtubular content was determined following a previously published procedure [29]. Briefly, samples of cell suspensions (200 μL; 2×10^6 cells/mL) were transferred to 200 μL of ice-cold 3% (w/v) formaldehyde in PBS for 10 min. Fixed cells were