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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

permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P40 detergent dissolved in PBS and then incubated for 30 min with Oregon Green 488 paclitaxel (1 μM) 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 488 nm and emission was at 522 nm.

2.7. Statistical analysis

Analysis of statistical significance was performed using one-way analysis of variance combined with the Dunnett tests. The difference was considered statistically significant when at least $p < 0.05$.

3. Results

3.1. The microtubules regulate TG-evoked SOCE and association between STIM1, Orai1 and TRPC1

In the absence of extracellular Ca^{2+}, the addition of 1 μM TG to fura-2-loaded HEK-293 cells in stirred cuvettes at 37 °C evoked a transient elevation in $[Ca^{2+}]_c$ due to the release of Ca^{2+} from intracellular stores. Subsequent addition of 2 mM Ca^{2+} to the external medium induced a sustained increase in $[Ca^{2+}]_c$ indicative of SOCE (the integral of the TG-evoked rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ was 15327 ± 1221 nM s; Fig. 1A). Treatment of HEK-293 cells with 1 μM TG resulted in association of Orai1 and TRPC1 with STIM1. Although the interaction of STIM1 with these two channels was detected in resting conditions, stimulation with TG enhanced the interaction between STIM1 and both Orai1 and TRPC1 by 75 ± 9% and 80 ± 11%, respectively (Fig. 1B and C; $p < 0.05$; $n = 6$).

We have investigated the role of microtubules in the activation of SOCE in HEK-293 cells and the interaction between STIM1, Orai1 and TRPC1 controlled by depletion of the intracellular stores by using colchicine, a tubulin microtubule disrupting agent [29,30], and paclitaxel that induces microtubular stabilization and increases the cellular microtubular content [31,32]. Treatment with 30 μM colchicine for 30 min at 37 °C significantly reduced the microtubular content in non-stimulated cells, as well as abolished tubulin polymerization after stimulation with 1 μM TG (Table 1; $p < 0.05$; $n = 6$). Cell treatment with colchicine enhanced TG-evoked SOCE by 23 ± 4% (the integral of the TG-evoked rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ in cells pretreated with colchicine was 18975 ± 543 nM s; $p < 0.05$), without having any effect on TG-evoked Ca^{2+} release from the intracellular stores (Fig. 1A). We further investigated the effect of treatment with colchicine on TG-induced association between STIM1 and both Orai1 and TRPC1. As shown in Fig. 1B and C, top panels, treatment with colchicine enhanced TG-evoked association between STIM1 and Orai1 by 75 ± 9% and between STIM1 and TRPC1 by 44 ± 6% ($p < 0.05$), without affecting the association between these proteins in non-stimulated cells ($n = 6$). Western blotting with the immunoprecipitating antibody confirms that a similar amount of protein was loaded in all lanes (Fig. 1B and C, bottom panels). Conversely, treatment of HEK-293 cells for 30 min with 10 μM paclitaxel at 37 °C enhanced the microtubular content in non-stimulated cells and prevented TG-evoked tubulin polymerization (Table 1; $p < 0.05$; $n = 6$). Treatment with paclitaxel attenuated TG-evoked SOCE by 60 ± 7% (the integral of TG-evoked rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ in cells pretreated with paclitaxel was 6238 ± 1508 nM s; $p < 0.05$), without having any effect on TG-evoked Ca^{2+} efflux from the stores (Fig. 2A). In addition, pretreatment with paclitaxel reduced TG-induced association between STIM1 and both Orai1 and TRPC1 by 49 ± 6% and 59 ± 5%, respectively ($p < 0.05$), without affecting the interaction between these proteins in non-stimulated cells (Fig. 2B and C, top panels; $n = 6$). Western blotting with the immunoprecipitating antibody confirms that a similar amount of protein was loaded in all lanes (Fig. 2B and C, bottom panels).

3.2. Jasplakinolide inhibits TG-evoked SOCE and association between STIM1, Orai1 and TRPC1

Jasplakinolide, a cell-permeant peptide isolated from *Jaspis johnstoni*, induces polymerization and stabilization of actin filaments underneath the plasma membrane [33]; therefore, it is a useful tool to explore the role of the cortical actin cytoskeleton in the activation of SOCE and its relevance in the association between STIM1 and the Ca^{2+} channels in the plasma membrane. Cell treatment for 30 min with 10 μM jasplakinolide at 37 °C resulted in a significant increase in the F-actin content in non-stimulated cells and prevented TG-induced further actin filament polymerization (Table 2; $p < 0.05$; $n = 6$). Treatment with jasplakinolide attenuated SOCE by 67 ± 7% (the integral of TG-evoked rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ in cells pretreated with jasplakinolide was 5118 ± 1867 nM s; $p < 0.05$), without having any effect on TG-evoked Ca^{2+} release from the intracellular stores (Fig. 3A). In cells treated with jasplakinolide TG was unable to enhance association between STIM1 and both Orai1 and TRPC1 (Fig. 3B and C, top panels; $p < 0.05$). Treatment with jasplakinolide did not affect the interaction between these proteins in cells not treated with TG (Fig. 3B and C, top panels). Western blotting with the anti-STIM1 antibody confirms a similar amount of protein loaded in all lanes (Fig. 3B and C, bottom panels).

3.3. Disruption of the actin cytoskeleton enhances TG-evoked SOCE

In order to investigate the role of the actin filament network in SOCE and TG-evoked association between STIM1 and the Ca^{2+} channels Orai1 and TRPC1, we used Cyt D, a widely utilized cell-permeant inhibitor of actin polymerization [17,34]. Pretreatment of HEK-293 cells for 40 min at 37 °C with 10 μM Cyt D reduced the F-actin content in non-stimulated cells and impaired TG-induced F-actin polymerization (Table 2; $p < 0.05$; $n = 6$). In addition, treatment with Cyt D enhanced TG-evoked Ca^{2+} entry by 71 ± 5% (the integral of the rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ was 25190 ± 1780 nM s in Cyt D-treated cells, Fig. 4A; $p < 0.05$; $n = 6$). In contrast to the effect of Cyt D on TG-evoked Ca^{2+} entry, treatment of HEK-293 cells with 10 μM Cyt D for 40 min at 37 °C did not significantly modify the association between STIM1 and both Orai1 and TRPC1 in non-stimulated cells or cells treated with TG (Fig. 4B and C, top panels). Western blotting with the immunoprecipitating antibody confirms a similar amount of protein loaded in all lanes (Fig. 4B and C, bottom panels).

3.4. Cytochalasin D impaires TG-stimulated association of calmodulin with Orai1 and TRPC1

Calmodulin has been previously reported to modulate Ca^{2+} signals and to interact with TRPC1 [35] and Orai1 [36]. Since disruption of the actin filament network by treatment with Cyt D reported different effects on TG-evoked SOCE and TG-evoked association between STIM1 and the Ca^{2+} channels, we further investigated the possible regulation of the association between calmodulin and the Ca^{2+} channels upon treatment with Cyt D. In a medium containing 1 mM Ca^{2+}, TG enhances the association between calmodulin and both Orai1 and TRPC1 proteins by 215 ± 47% and 121 ± 27%, respectively (Fig. 5A and B, top panels; $p < 0.05$; $n = 6$). Treatment of HEK-293 cells for 40 min with 10 μM Cyt D did not significantly modify the association between calmodulin and the channel proteins in non-stimulated cells but abolished TG-evoked responses (Fig. 5A and B, top panels; $p < 0.05$;

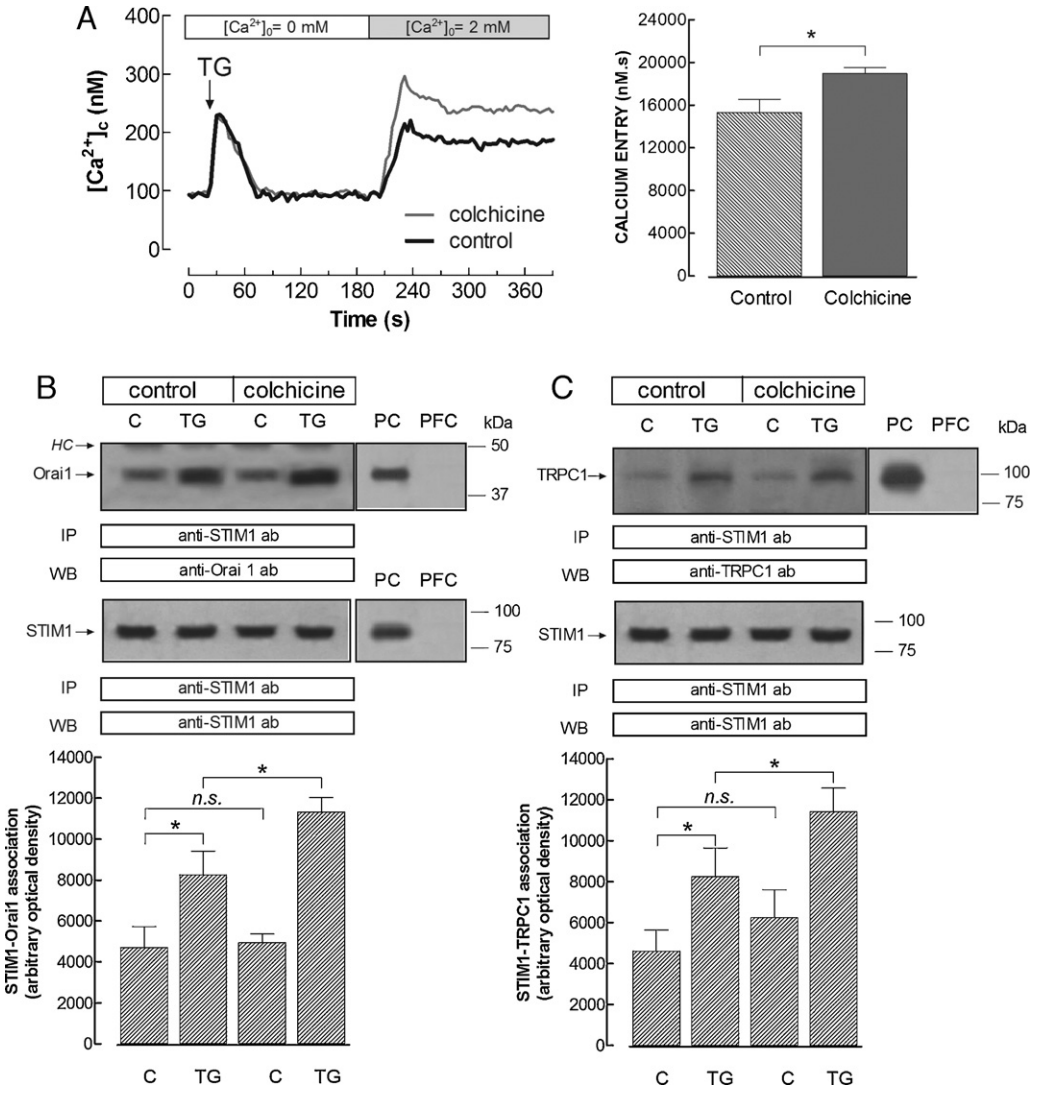

Fig. 1. Effect of colchicine on TG-evoked Ca^{2+} entry and association between STIM1 and both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated for 30 min in the absence or presence of 30 μM colchicine. At the time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3 min later $CaCl_2$ (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate Ca^{2+} entry in the presence or absence of colchicine, as indicated. Ca^{2+} entry was determined as described in Section 2. Values are means ± S.E.M. of six independent experiments; $^*p < 0.05$. (B and C) HEK-293 cells were preincubated in presence of 30 μM colchicine or the vehicle (Control), as indicated, suspended in a Ca^{2+}-free medium (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were incubated overnight with 2 μg/mL anti-STIM1 antibody and 25 μg/mL protein A-agarose and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes were reprobed with the anti-STIM1 antibody for protein loading control (bottom panels). The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of STIM1–Orai1 or STIM1–TRPC1 association in non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical density units and expressed as means ± S.E.M. $^*p < 0.05$, n.s., non significant. PC and PFC, primary control and primary-free control, respectively. For PC, whole cell lysates were subjected to 10% SDS-PAGE and subsequent Western blotting with the corresponding primary and secondary antibodies. For PFC, whole cell lysates were subjected to 10% SDS-PAGE and the membrane was subsequently incubated solely with the secondary antibody. HC, heavy chain of the immunoglobulin used for immunoprecipitation.

Table 1
Effects of colchicine and paclitaxel on the microtubular content of unstimulated and TG-stimulated HEK-293 cells.

Stimulatory agent	Control	Colchicine	Paclitaxel
None	100 ± 0	47 ± 5†	139 ± 4†
TG	127 ± 3*	51 ± 7†	145 ± 5†

HEK-293 cells were incubated with 30 μM colchicine or 10 μM paclitaxel for 30 min or the vehicles for the same period as controls. Cells were suspended in a Ca^{2+}-free medium (1.2 mM EGTA added) and then treated with 1 μM TG. Samples were removed 5 s before and 3 min after adding TG and the microtubular content was determined as described in material and methods. Values given are the microtubular content expressed as a percentage of the basal content and are presented as means ± S.E.M. of six separate determinations.

* $p < 0.01$ compared with the microtubular content in non-stimulated cells.

† $p < 0.05$ compared with the microtubular content in cells treated in the absence of inhibitors.

$n = 6$). Western blotting with the immunoprecipitating antibody confirms a similar amount of protein loaded in all lanes (Fig. 5A and B, bottom panels). In order to investigate whether TG-evoked Ca^{2+} release from the stores is sufficient to activate association between calmodulin and the Ca^{2+} channel proteins Orai1 and TRPC1, we repeated the experiments in a Ca^{2+}-free medium. In the absence of extracellular Ca^{2+} treatment with TG significantly enhanced Orai1-calmodulin and TRPC1-calmodulin interaction by 169 ± 26% and 45 ± 10%, respectively, as detected by co-immunoprecipitation (Fig. 5C and D, top panels; $p < 0.05$; $n = 6$). TG-induced association between calmodulin and both Orai1 and TRPC1 was significantly greater when the experiments were performed in a medium containing 1 mM Ca^{2+}, which allowed Ca^{2+} to enter (Fig. 5, top panels; $p < 0.05$). TG-evoked association between calmodulin and both Orai1 and TRPC1 was abolished by treatment with Cyt D (Fig. 5C

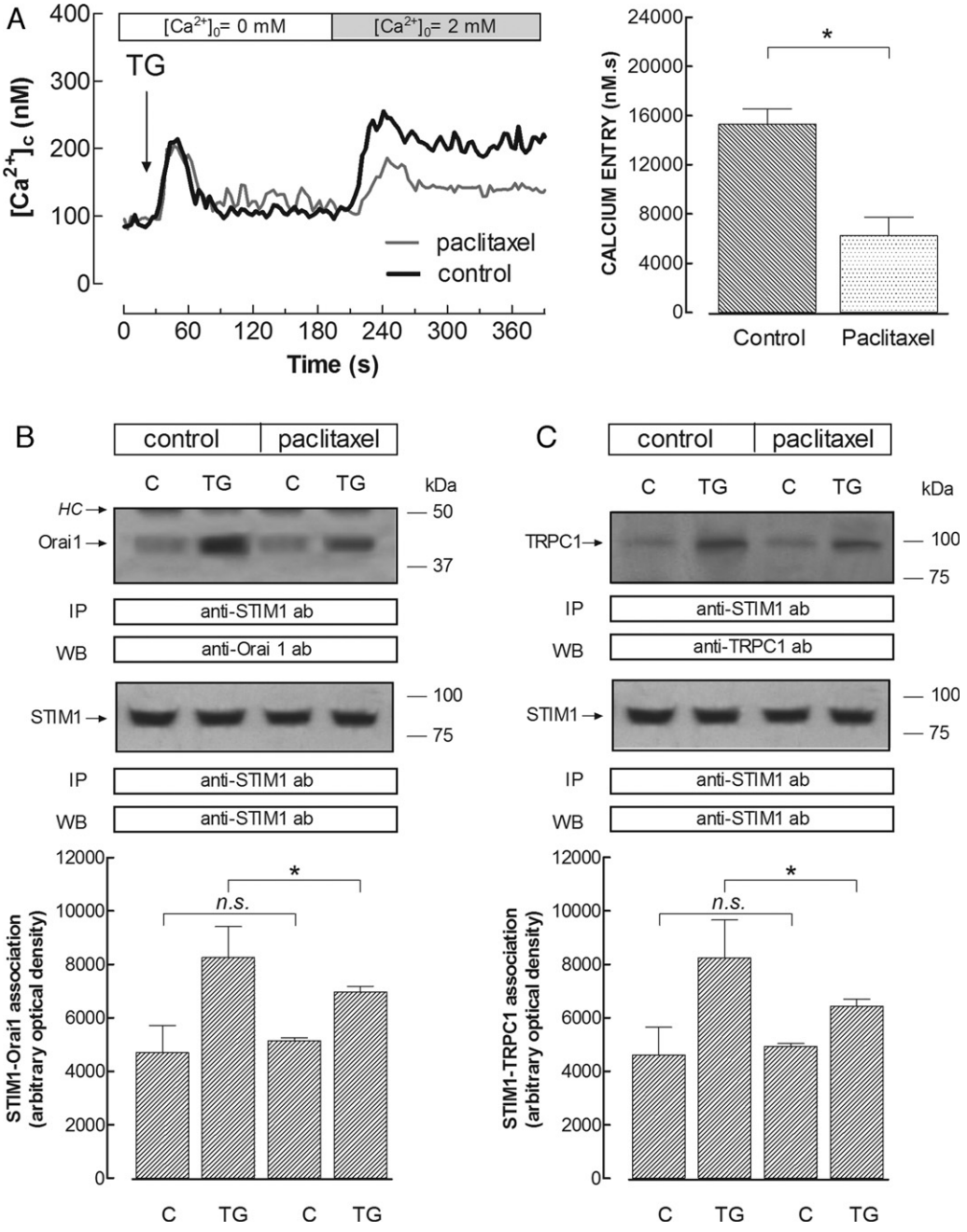

Fig. 2. Effect of paclitaxel on TG-evoked Ca^{2+} entry and association between STIM1 and both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated for 30 min in the absence or presence of 10 μM paclitaxel. At the time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3 min later $CaCl_2$ (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate Ca^{2+} entry in the presence or absence of paclitaxel, as indicated. Ca^{2+} entry was determined as described in Section 2. Values are means ± S.E.M. of six independent experiments; $^*p < 0.05$. (B and C) HEK-293 cells were preincubated in presence of 10 μM paclitaxel or the vehicle (Control), as indicated, suspended in a Ca^{2+}-free medium (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were incubated overnight with 2 μg/mL anti-STIM1 antibody and 25 μg/mL protein A-agarose and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes were reprobed with the anti-STIM1 antibody for protein loading control (bottom panels). The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of STIM1–Orai1 or STIM1–TRPC1 association in non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical density units and expressed as means ± S.E.M. $^*p < 0.05$, n.s., non significant. HC, heavy chain of the immunoglobulin used for immunoprecipitation.

and D; $p < 0.05$; $n = 6$), thus suggesting that this association requires the integrity of the actin filament network.

Previous studies have reported that calmodulin antagonists potentiate SOCE in pulmonary artery smooth muscle cells [37], and cerebellar granule cells [38] and astrocytes [39]. Hence, we have further investigated the effect of the calmodulin antagonist calmidazolium on TG-evoked Ca^{2+} entry in HEK-293 cells. Our results indicate that treatment for 10 min with 1 μM calmidazolium enhances TG-induced calcium entry by 63 ± 7% (the integral of the rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ was 25092 ± 356 nM s in calmidazolium-treated cells), without inducing significant changes in TG-evoked Ca^{2+} release (Fig. 6; $p < 0.05$; $n = 6$). The effect of calmidazolium was similar to that observed when cells were pretreated with calmidazolium in the presence of Cyt D, which enhanced TG-evoked SOCE by 69 ± 5% (the integral of the rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ was 26034 ± 249 nM s in calmidazolium + Cyt D-treated cells; Fig. 6; $p < 0.05$; $n = 6$); thus indicating that the effect of Cyt D enhancing TG-evoked Ca^{2+} entry

Table 2
Effects of Cyt D and jasplakinolide on the F-actin content of unstimulated and TG-stimulated HEK-293 cells.

Stimulatory agent	Control	Cyt D	Jasplakinolide
None	100 ± 0	42 ± 7†	158 ± 7†
TG	141 ± 5*	39 ± 6†	163 ± 6†

HEK-293 cells were incubated with 10 μM cytochalasin D for 40 min, 10 μM jasplakinolide for 30 min or the vehicles for the same period as controls. Cells were suspended in a Ca^{2+}-free medium (1.2 mM EGTA added) and then treated with 1 μM TG. Samples were removed 5 s before and 3 min after adding TG and the F-actin content was determined as described in material and methods. Values given are the F-actin content expressed as a percentage of the basal content and are presented as means ± S.E.M. of six separate determinations.

* $p < 0.01$ compared with the F-actin content in non-stimulated cells.

† $p < 0.05$ compared with the F-actin content in cells treated in the absence of inhibitors.

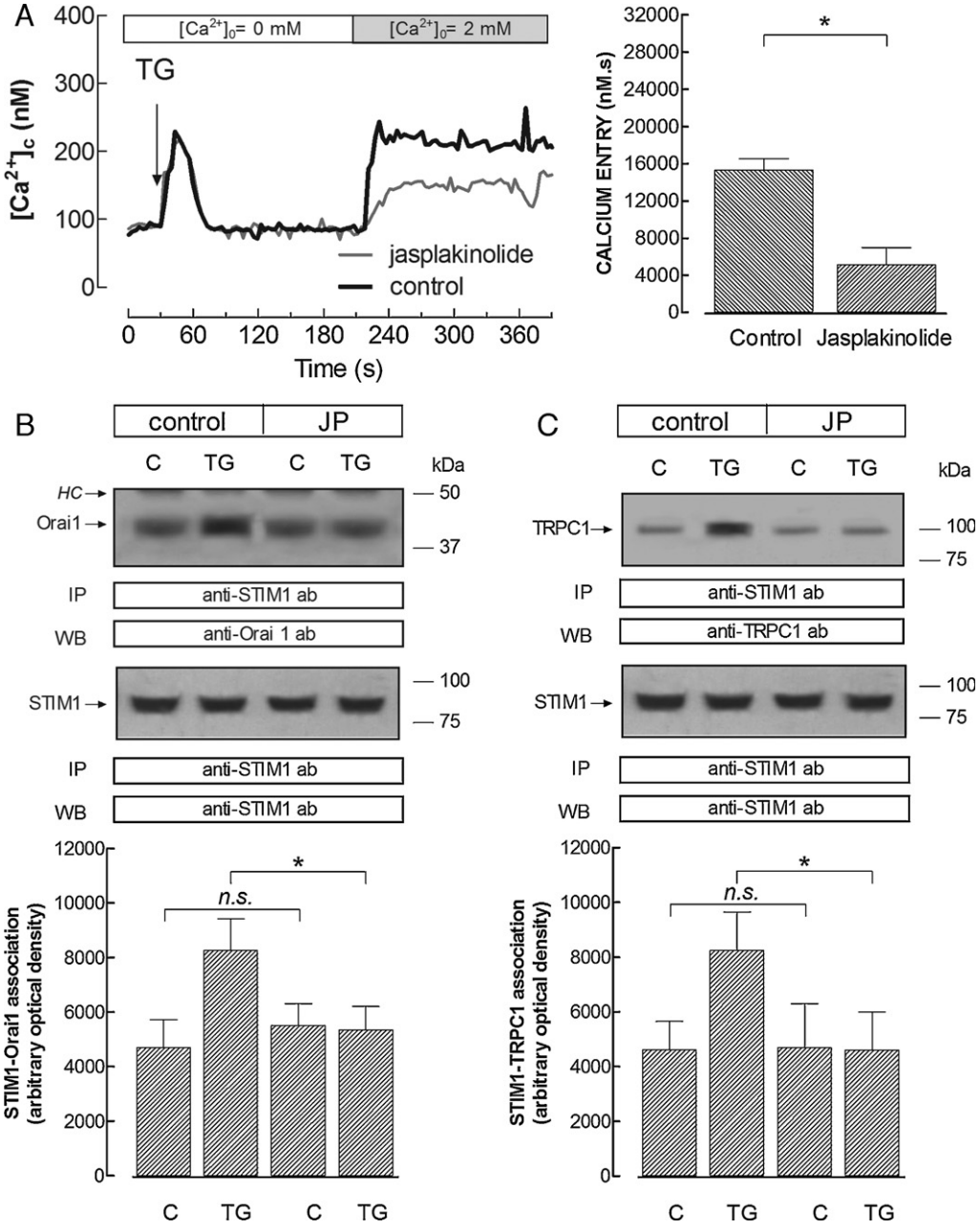

Fig. 3. Effect of jasplakinolide on TG-evoked Ca^{2+} entry and association between STIM1 and both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated for 30 min in the absence or presence of 10 μM jasplakinolide. At the time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3 min later $CaCl_2$ (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate Ca^{2+} entry in the presence or absence of jasplakinolide, as indicated. Ca^{2+} entry was determined as described in Section 2. Values are means ± S.E.M. of six independent experiments; * $p < 0.05$. (B and C) HEK-293 cells were preincubated in presence of 10 μM jasplakinolide or the vehicle (Control), as indicated, suspended in a Ca^{2+}-free medium (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were incubated overnight with 2 μg/mL anti-STIM1 antibody and 25 μg/mL protein A-agarose and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes were reprobed with the anti-STIM1 antibody for protein loading control (bottom panels). The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of STIM1–Orai1 or STIM1–TRPC1 association in non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical density units and expressed as means ± S.E.M. * $p < 0.05$, n.s., non significant. HC, heavy chain of the immunoglobulin used for immunoprecipitation.

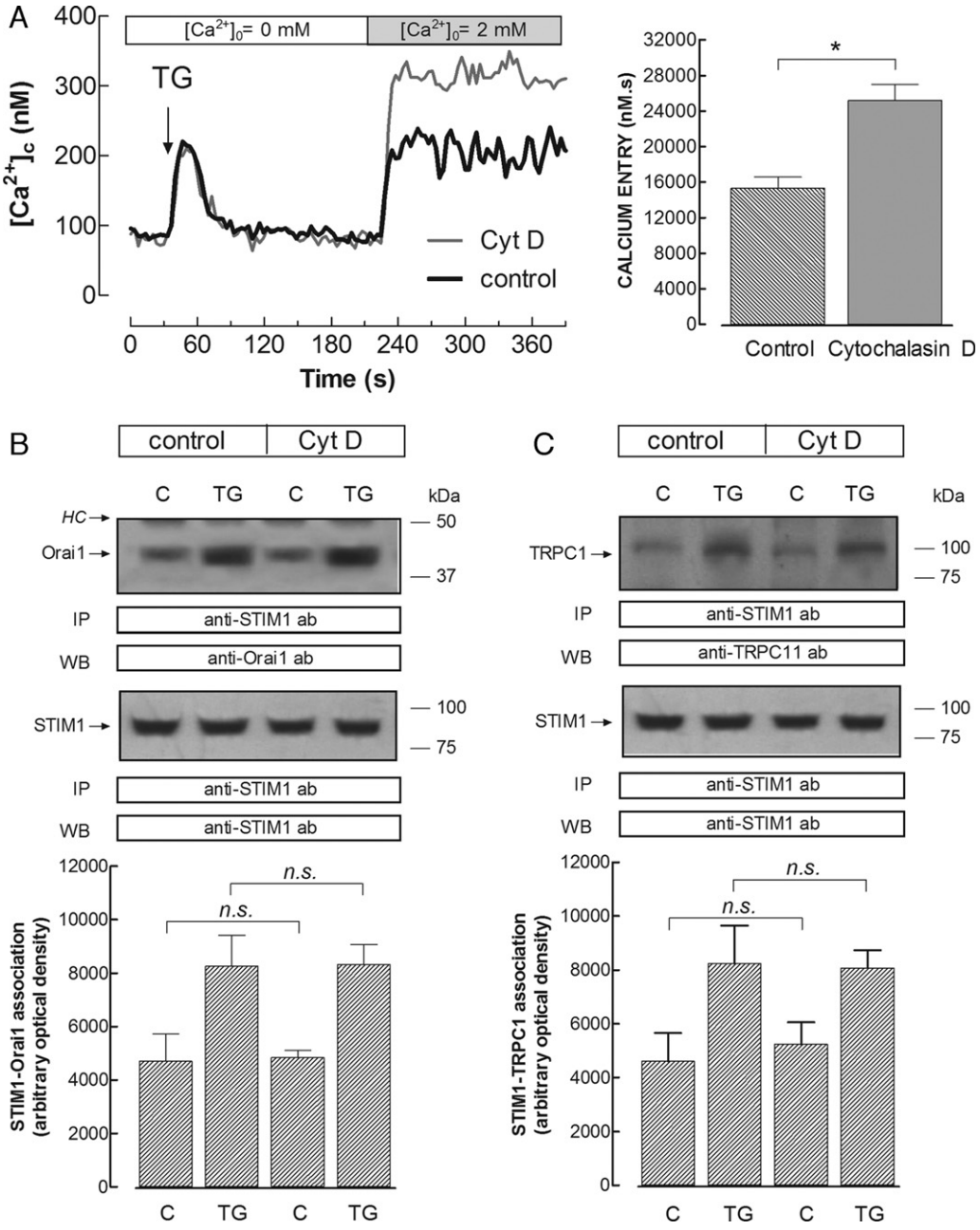

Fig. 4. Effect of cytochalasin D on TG-evoked Ca^{2+} entry and association between STIM1 and both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated for 40 min in the absence or presence of 10 μM Cyt D. At the time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3 min later $CaCl_2$ (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate Ca^{2+} entry in the presence or absence of cytochalasin D, as indicated. Ca^{2+} entry was determined as described in Section 2. Values are means ± S.E.M. of six independent experiments; $^*p < 0.05$. (B and C) HEK-293 cells were preincubated in presence of 10 μM Cyt D or the vehicle (Control), as indicated, suspended in a Ca^{2+}-free medium (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were incubated overnight with 2 μg/mL anti-STIM1 antibody and 25 μg/mL protein A-agarose and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes were reprobed with the anti-STIM1 antibody for protein loading control. The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of STIM1–Orai1 or STIM1–TRPC1 association in non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical density units and expressed as means ± S.E.M. n.s., non significant. HC, heavy chain of the immunoglobulin used for immunoprecipitation.

might be attributed to inhibition of the regulatory role of calmodulin on SOCE.

4. Discussion

The role of the cytoskeleton on the activation of Ca^{2+} entry following the depletion of intracellular Ca^{2+} stores is a complex signalling process of great relevance that has been investigated in a number of cells providing discrepant results. In human platelets, we have found that microtubules play a dual role in SOCE, acting as a barrier that prevents constitutive SOCE regulated by the dense tubular system, the ER analogue in platelets, and also supporting SOCE mediated by the acidic Ca^{2+} stores [19]. In contrast, in HEK-293 cells microtubules seems to support SOCE and I_{CRAC}, which has been attributed to a role of the microtubules in STIM1 localization [40]. On the other hand, SOCE has been reported to be insensitive to Cyt D, a widely used membrane-permeant inhibitor of actin polymerization, in a number of cells, such as the DDT_1MF-2

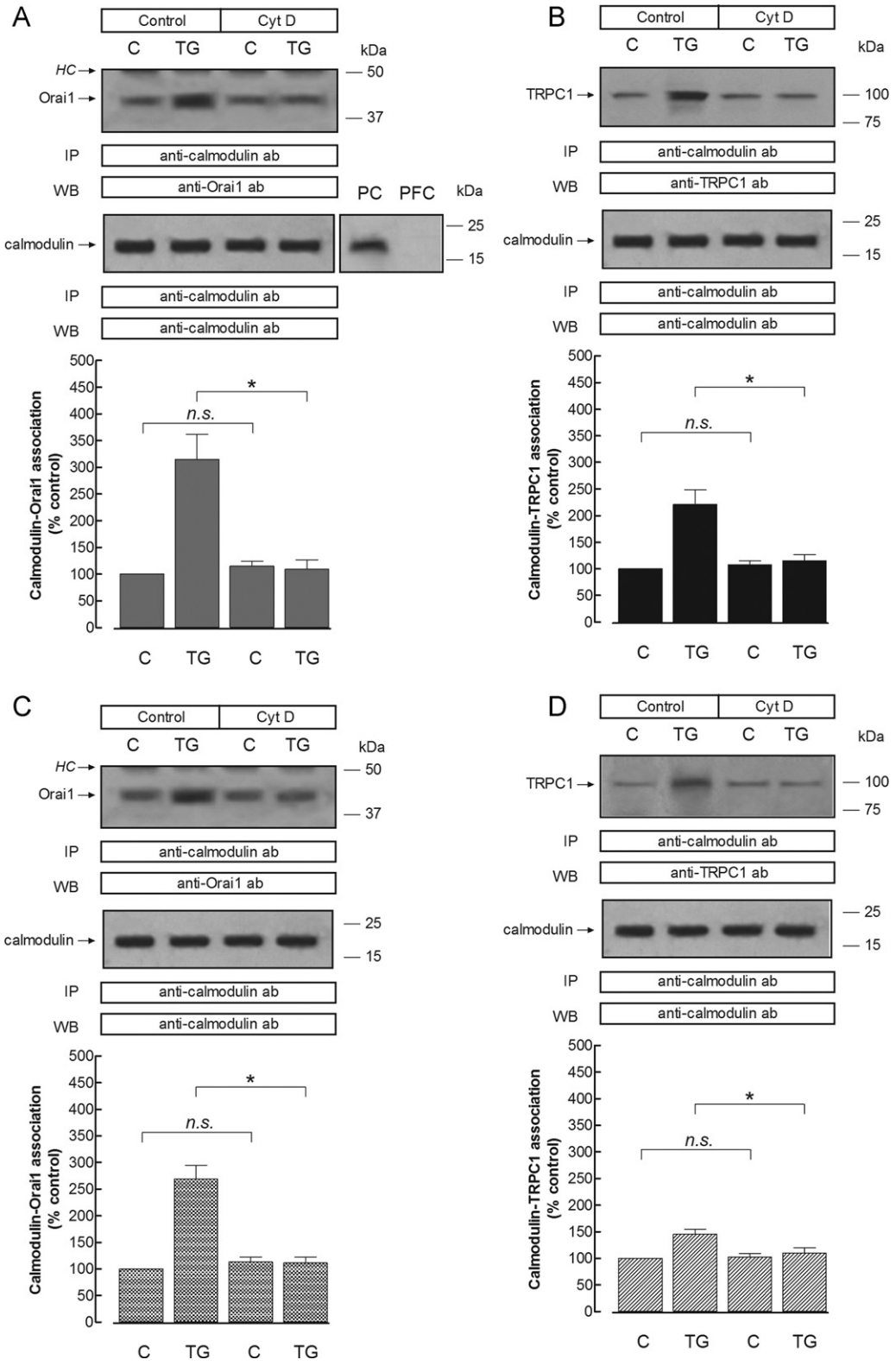

Fig. 5. Effect of cytochalasin D on TG-evoked association between calmodulin and both Orai1 and TRPC1 in HEK-293 cells. HEK-293 cells (2×10^6 cells/mL) were preincubated in presence of 10 μM Cyt D or the vehicle (Control), as indicated, and either suspended in a medium containing 1 mM Ca^{2+} (A and B) or suspended in a Ca^{2+}-free medium (1.2 mM EGTA added, C and D). Cells were then stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were incubated overnight with 2 μg/mL anti-calmodulin antibody and 25 μg/mL protein A-agarose and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai1 or anti-TRPC1 antibody, as indicated. Membranes were reprobed with the anti-STIM1 antibody for protein loading control (bottom panels). The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of calmodulin–Orai1 or calmodulin–TRPC1 association in non-stimulated (control) and TG-treated cells. Results are presented as percentage of control and expressed as means ± S.E.M. $^*p < 0.05$, n.s., non significant. HC, heavy chain of the immunoglobulin used for immunoprecipitation. PC and PFC, primary control and primary-free control, respectively. For PC, whole cell lysates were subjected to 10% SDS-PAGE and subsequent Western blotting with the corresponding primary and secondary antibodies. For PFC, whole cell lysates were subjected to 10% SDS-PAGE and the membrane was subsequently incubated solely with the secondary antibody.

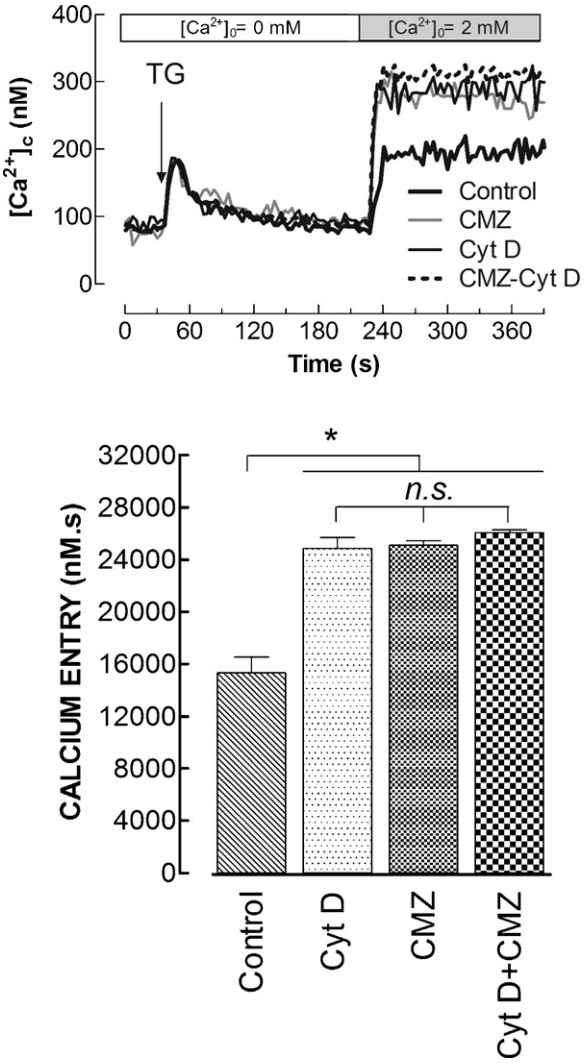

Fig. 6. Effect of calmidazolium and/or cytochalasin D on TG-evoked Ca^{2+} entry in HEK-293 cells. Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated either for 40 min with 10 μM Cyt D, for 10 min with 1 μM calmidazolium, both or the vehicle as control. At the time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3 min later $CaCl_2$ (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate Ca^{2+} entry in the presence or absence of Cyt D, calmidazolium or both, as indicated. Ca^{2+} entry was determined as described in materials and methods. Values are means ± S.E.M. of six independent experiments; $^{*}p < 0.05$. n.s., non significant.

transformed smooth muscle cell line and the nontransformed A7r5 smooth muscle line derived from rat embryonic aorta [14]. In contrast, the actin network plays an important role in SOCE in neutrophils [41], human keratinocytes [42], bovine adrenocortical fasciculata cells [43], glioma C6 cells [44], vascular endothelial cells [20] and human platelets [17,18,45]. In the latter, we have reported a time-dependent effect of Cyt D on platelet SOCE, which is consistent with a dual role for the actin cytoskeleton in SOCE both acting as a negative clamp that prevents constitutive SOCE but also supporting the coupling between elements in the membrane of the ER and Ca^{2+} channels in the plasma membrane [17,18].

We have found that disruption of the actin cytoskeleton by Cyt D or treatment with the microtubule disrupting agent, colchicine, increases TG-evoked SOCE, while stabilization of the cortical actin network or the microtubules using jasplakinolide and paclitaxel, respectively, basically induced opposite effects, attenuating TG-evoked SOCE, as expected. These observations suggest that the actin cytoskeleton and microtubules play an important role in Ca^{2+} influx, probably acting as a barrier that prevents SOCE in resting cells, as reported in human platelets [19]. A previous study has reported that nocodazole and colchicine reduce TG-evoked SOCE in HEK-293 cells [40]. This apparent contradiction between our observations and those reported by Smyth et al. might be attributed to the fact that both studies estimate SOCE at different stages. In our study, the initiation of Ca^{2+} entry was induced 3 min after the addition of TG, while in the study published by Smyth et al. TG was added to cells suspended in a medium containing Ca^{2+} [40]. Different stages for SOCE have been reported in different cell types, such as pancreatic acinar cells [46] and human platelets [47], the latter differentially regulated by the cytoskeleton [48].

The role of the cytoskeleton in the modulation of SOCE has been attributed to a number of signalling events, including the facilitation of STIM1 localization within the ER [40], probably through its interaction with the microtubule-plus-end-tracking protein EB1 [49], the regulation of TRP channels internalization [41] or by modulating the interaction of TRPC proteins with the type II IP_3 receptor [50,51]. Here, we report for the first time that the cytoskeleton regulates the association of STIM1 with Orai1 and the TRPC1 channels in the plasma membrane. The reported effects of the microtubule altering manoeuvres, either using colchicine or paclitaxel, or those of jasplakinolide, on SOCE are paralleled by a similar modification of the association between STIM1 and the Ca^{2+} channels, and suggest that both the actin filaments and microtubules act as a cortical barrier that prevents the association between STIM1 and the Ca^{2+} channels, as well as SOCE (see Fig. 7), thus disruption of this barrier facilitates the coupling between STIM1 and the Ca^{2+} channels, as well as SOCE, while stabilization of the cortical actin or microtubular networks impairs both events. However, we found that actin filament disruption enhances SOCE without inducing any significant effect on the association between STIM1 and Orai1 or STIM1 and TRPC1. Interestingly, we have found that microfilament disruption results in inhibition of TG-induced association between calmodulin and both Orai1 and TRPC1 (see Fig. 7). Since inhibition of calmodulin results in a similar increase in TG-evoked SOCE than disruption of the actin network our results provide evidence for a role of calmodulin in the regulation of SOCE through its interaction with Orai1 and TRPC1 supported by the actin cytoskeleton. The microtubules must play a further important regulatory role in the association of STIM1 with the Ca^{2+} channels as previously reported [12], since disruption of the microtubules enhances this association, an event that has not been found after inhibition of actin polymerization.

Calmodulin has been reported to interact with TRP proteins, including *Drosophila* TRPL [52] and mammalian TRPC1 [35]. More recently, calmodulin was found to interact with the polybasic C-termini of STIM1 [53] and with a membrane-proximal N-terminal domain of Orai1 [36] in a Ca^{2+} dependent manner. Different experimental manoeuvres have demonstrated that calmodulin is involved in Ca^{2+}-dependent inactivation of SOCE, including expression of TRPC1 or Orai1 mutants lacking the ability to bind calmodulin, which prevented Ca^{2+}-dependent inactivation of SOCE in human salivary gland cells [35], or I_{CRAC} in HEK-293 cells [36], respectively, or over-expression of a calmodulin inhibitor peptide and a Ca^{2+}-insensitive calmodulin mutant, which results in reduced inactivation of I_{SOC} in liver cells [54]. Our results indicate that association of calmodulin with the Ca^{2+} channels Orai1 and TRPC1 requires the integrity of the actin cytoskeleton, which might provide a support for these associations, and, therefore, disruption of the actin network leads to impairment of Ca^{2+}-dependent inactivation of SOCE reported above.

We have not performed experiments to assess whether the effect of the cytoskeleton modifiers is maximal; however, the changes induced in the microtubules and actin filaments are sufficiently evident to demonstrate that the cytoskeleton plays a

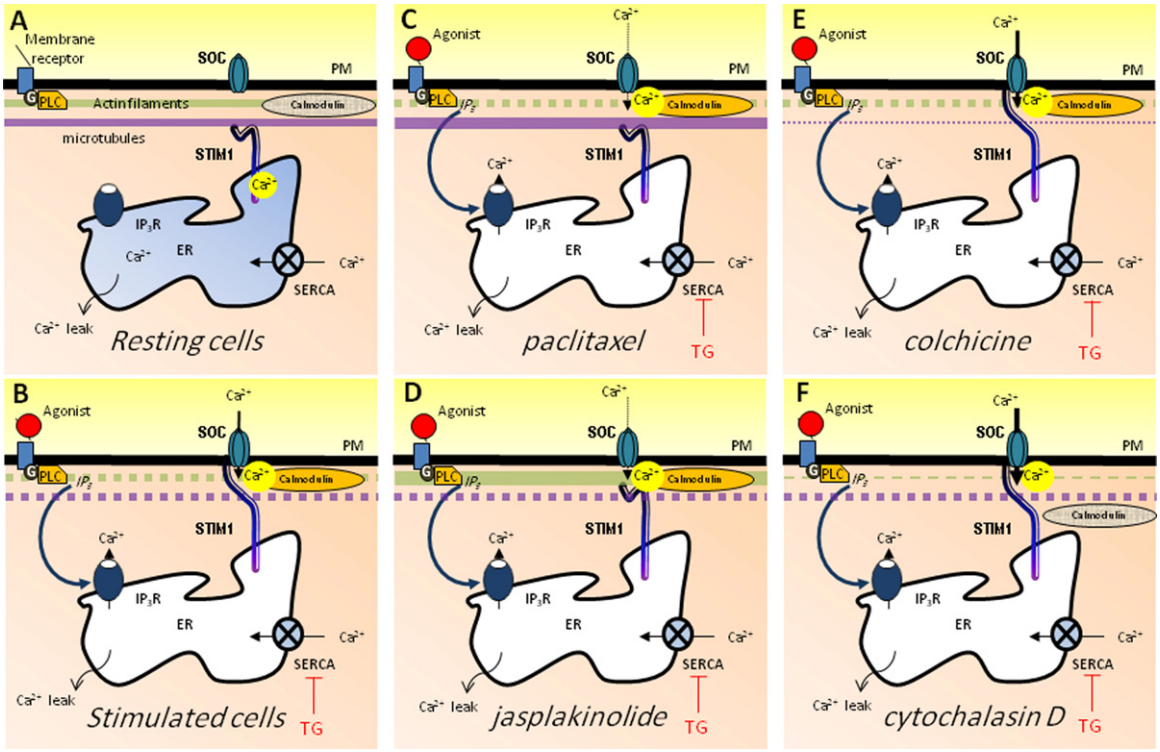

Fig. 7. Proposed model for cytoskeletal remodelling during SOCE in HEK-293 cells. (A) In the resting state the Ca^{2+} stores are full and the actin filaments and microtubules, located beneath the plasma membrane, act as a cortical barrier preventing constitutive association between STIM1 and SOC channels. (B) Cell stimulation with physiological agonists (or inhibition of Ca^{2+} reuptake by SERCA using thapsigargin (TG)) results in discharge of the Ca^{2+} stores leading to remodelling of the actin and microtubular networks to facilitate the association of STIM1 with SOC channels and the activation of SOCE. (C and D) Stabilization of the actin or microtubular peripheral network prevents the association of STIM1 with SOC channels and SOCE. (E) Disruption of the microtubular barrier facilitates agonist (or store-depletion)-induced association events and SOCE. (F) Disruption of the actin filament network enhances agonist-stimulated SOCE by impairing the association of calmodulin with SOC channels. ER, endoplasmic reticulum; G, heterotrimeric G protein; IP_3, inositol 3,4,5-trisphosphate; IP_3R, IP_3 receptor; PLC, phospholipase C; PM, plasma membrane; SOC, store-operated Ca^{2+} channels (i.e., Orai1 and TRPC1).

relevant role in the regulation of SOCE in HEK-293 cells through the regulation of the interaction between STIM1 and Orai1 or TRPC1. In addition, the actin cytoskeleton supports the association of calmodulin with the Ca^{2+} channels in the plasma membrane, which provides an additional regulatory role of store-operated Ca^{2+} influx.

Conflict of interest

The authors state that there is no conflict of interest.

Acknowledgements

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