



HAL
open science

The cytoskeleton plays a modulatory role in the association between STIM1 and the Ca channel subunits Orai1 and TRPC1.

Carmen Galán, Natalia Dionisio, Tarik Smani, Ginés M. Salido, Juan A. Rosado

► To cite this version:

Carmen Galán, Natalia Dionisio, Tarik Smani, Ginés M. Salido, Juan A. Rosado. The cytoskeleton plays a modulatory role in the association between STIM1 and the Ca channel subunits Orai1 and TRPC1.. *Biochemical Pharmacology*, 2011, 82 (4), pp.400. 10.1016/j.bcp.2011.05.017 . hal-00718033

HAL Id: hal-00718033

<https://hal.science/hal-00718033>

Submitted on 16 Jul 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The cytoskeleton plays a modulatory role in the association between STIM1 and the Ca²⁺ channel subunits Orai1 and TRPC1.

Authors: Carmen Galán, Natalia Dionisio, Tarik Smani, Ginés M. Salido, Juan A. Rosado



PII: S0006-2952(11)00330-3
DOI: doi:10.1016/j.bcp.2011.05.017
Reference: BCP 10909

To appear in: *BCP*

Received date: 25-2-2011
Revised date: 15-5-2011
Accepted date: 18-5-2011

Please cite this article as: Galán C, Dionisio N, Smani T, Salido GM, Rosado JA, The cytoskeleton plays a modulatory role in the association between STIM1 and the Ca²⁺ channel subunits Orai1 and TRPC1., *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2011.05.017

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1
2
3
4 The cytoskeleton plays a modulatory role in the association between
5
6
7 STIM1 and the Ca²⁺ channel subunits Orai1 and TRPC1.
8
9

10
11 Carmen Galán[†], Natalia Dionisio[†], Tarik Smani*, Ginés M. Salido[†], Juan A.
12
13 Rosado[†]
14

15
16 [†]*Department of Physiology (Cellular Physiology Research Group), University of*
17
18 *Extremadura, 10071 Cáceres, and *Institute of Biomedicine from Seville, University of*
19
20 *Hospital of Virgen del Rocío, University of Seville, Spain*
21
22
23
24
25
26

27 *Running head:* The cytoskeleton modulates STIM1-Ca²⁺ channels interaction
28

29
30 *Category:* Metabolic Disorders and Endocrinology.
31
32
33
34
35

36 To whom correspondence should be addressed:
37

38 Dr. J. A. Rosado,
39

40 Department of Physiology,
41

42 University of Extremadura,
43

44 Cáceres 10003, SPAIN
45

46 Tel: +34 927 257139
47

48 Fax: +34 927257110
49

50
51 E-mail: jarosado@unex.es
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3 **ABSTRACT**
4
5

6 Store-operated Ca^{2+} entry (SOCE) is a major pathway for Ca^{2+} influx in non-excitabile cells.
7
8 Recent studies favour a conformational coupling mechanism between the endoplasmic
9 reticulum (ER) Ca^{2+} sensor STIM1 and Ca^{2+} permeable channels in the plasma membrane to
10 explain SOCE. Previous studies have reported a role for the cytoskeleton modulating the
11 activation of SOCE; therefore, here we have investigated whether the interaction between
12 STIM1 and the Ca^{2+} permeable channels is modulated by the actin or microtubular network.
13
14 In HEK-293 cells, treatment with the microtubular disrupter colchicine enhanced both the
15 activation of SOCE and the association between STIM1 and Orai1 or TRPC1 induced by
16 thapsigargin (TG). Conversely, stabilization of the microtubules by paclitaxel attenuated TG-
17 evoked activation of SOCE and the interaction between STIM1 and the Ca^{2+} channels Orai1
18 and TRPC1, altogether suggesting that the microtubules act as a negative regulator of SOCE.
19
20 Stabilization of the cortical actin filament layer results in inhibition of TG-evoked both
21 association between STIM1, Orai1 and TRPC1 and SOCE. Interestingly, disruption of the
22 actin filament network by cytochalasin D did not significantly modify TG-evoked association
23 between STIM1 and Orai1 or TRPC1 but enhanced TG-stimulated SOCE. Finally, inhibition
24 of calmodulin by calmidazolium enhances TG-evoked SOCE and disruption of the actin
25 cytoskeleton results in inhibition of TG-evoked association of calmodulin with Orai1 and
26 TRPC1. Thus, we demonstrate that the cytoskeleton plays an essential role in the regulation
27 of SOCE through the modulation of the interaction between their main molecular
28 components.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54

55
56
57 *Keywords:* actin filaments, microtubules, calmodulin, TRPC1, STIM1, Orai1.
58
59
60
61
62
63
64
65

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 HEK293 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

1 type. In NIH 3T3 fibroblasts and smooth muscle DT40 cells inhibition of actin
2 polymerization with cytochalasin or depolymerisation of the microtubular network with
3 nocodazole does not significantly alter SOCE [13-16], thus suggesting that in these cells the
4 cytoskeleton is not essential for the activation of SOCE. In contrast, in human platelets,
5 which have a more evenly distributed cytoskeleton, impairment of actin filaments and
6 microtubules remodelling either by inhibition of polymerization or by stabilization results in
7 inhibition of SOCE [17-19] and a similar functional role has been described for the actin
8 cytoskeleton in endothelial cells [20] and pancreatic acinar cells [21]. In human platelets we
9 have more recently reported that store depletion results in an initial decrease in the actin
10 filament content, probably due to disorganization of the cortical actin network to facilitate
11 the interaction between proteins in the ER and plasma membrane, followed by an increase in
12 actin polymerization necessary for the intracellular trafficking of portions of the ER towards
13 the plasma membrane [22]. Therefore, we have explored the functional relevance of the
14 cytoskeleton in the association between STIM1 and the SOC components Orai1 and TRPC1
15 upon depletion of the intracellular Ca^{2+} stores in HEK-293 cells endogenously expressing
16 these proteins.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 (Franklin 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 was 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 (HEK293) cells were obtained from the American Type Culture Collection (Barcelona, Spain) and cultured in Dulbecco's modified Eagle's medium,

1 supplemented with 10% heat-inactivated fetal bovine serum, in a 37 °C incubator with 5%
2 CO₂. At the time of the experiments cells were suspended in HEPES-buffered saline (HBS)
3 containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO₄, 1 mM CaCl₂, pH
4 7.45. When a Ca²⁺-free medium was required 1.2 mM EGTA was added.
5
6
7
8
9

10 2.3. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_c$)

11
12
13 HEK293 cells were suspended and loaded with fura-2 by incubation with 2 μM fura-
14 2/AM and 2.5 mM probenecid for 30 min at 37 °C. Fluorescence was recorded from 2 mL
15 aliquots of magnetically stirred cellular suspension (2×10^6 cells/mL) at 37°C using a Cary
16 Eclipse spectrophotometer (Varian Ltd, Madrid, Spain) with excitation wavelengths of 340
17 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2
18 340/380 fluorescence ratio and calibrated in terms of $[Ca^{2+}]_c$ [23].
19
20
21
22
23
24
25
26
27
28

29 Ca²⁺ release by TG was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after
30 the addition of the agonist. Ca²⁺ entry into TG-treated cells upon addition of Ca²⁺ to the
31 extracellular medium was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after
32 addition of CaCl₂ ($\int \Delta[Ca^{2+}]_c dt$), normalized taking a sample every second, and expressed as
33 nM•s [24-26]. Ca²⁺ entry was corrected by subtraction of the $[Ca^{2+}]_c$ elevation due to leakage
34 of the indicator to eliminate interferences due to extracellular fura-2.
35
36
37
38
39
40
41
42
43
44

45 2.4. Immunoprecipitation and Western blotting

46
47
48 The immunoprecipitation and Western blotting were performed as described previously
49 [27]. Briefly, Cell suspension aliquots (500 μL; 2×10^6 cells/mL) were treated as described
50 and lysed with lysis buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA,
51 0.2% SDS, 2% sodium deoxycholate, 2% triton X-100, 2 mM phenyl methyl sulfonyl
52 fluoride, 100 μg/mL leupeptin and 10 mM benzamidine. Samples were immunoprecipitated
53
54
55
56
57
58
59
60
61
62
63
64
65

1 by simultaneous incubation with 2 μg of anti-STIM1 antibody and protein A-agarose
2 overnight at 4°C on a rocking platform. Proteins were separated by 10% SDS-PAGE and
3 electrophoretically transferred, for 2 h at 0.8 mA/cm², in a semi-dry blotter (Hoefer
4 Scientific, Newcastle, Staffs., U.K.) onto nitrocellulose membranes for subsequent probing.
5 Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1%
6 Tween 20 (TBST) to block residual protein binding sites. Immunodetection of Orai1,
7 hTRPC1, calmodulin and STIM1 was achieved using the anti-Orai1, anti-calmodulin or anti-
8 STIM1 antibodies diluted 1:1000 in TBST for 2h or the anti-hTRPC1 antibody diluted 1:200
9 in TBST for 2h. To detect the primary antibody, blots were incubated for 1h with the
10 appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:10000 in TBST
11 and then exposed to enhanced chemiluminescence reagents for 5 min. Blots were then
12 exposed to photographic films. The density of bands on the film was measured using Image J
13 (Windows version; National Institutes of Health) [28].
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

32
33 To assess the specificity of the bands we performed primary controls and primary-free
34 controls, where whole cell lysates (2×10^6 cells/mL) were subjected to 10% SDS/PAGE and
35 proteins were electrophoretically transferred onto membranes for incubation with primary
36 and subsequently secondary antibody (primary control) or incubation solely with secondary
37 antibody (primary-free control).
38
39
40
41
42
43
44
45

46 2.5. Measurement of F-actin Content

47
48 The F-actin content was determined according to a previously published procedure [22].
49 Briefly, samples of cell suspensions (200 μL ; 2×10^6 cells/mL) were transferred to 200 mL
50 of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS; composition, in mM:
51 NaCl 137, KCl 2.7, Na₂HPO₄ 4.3, KH₂PO₄ 1.4, pH 7.3) for 10 min. Fixed cells were
52 permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved
53
54
55
56
57
58
59
60
61
62
63
64
65

1 in PBS. Cells were then incubated for 30 min with 1 μ M FITC-labeled phalloidin in PBS
2
3 supplemented with 0.5% (w/v) BSA. Cells were collected by centrifugation and resuspended
4
5 in PBS. Cell staining was measured using a Perkin-Elmer fluorescence spectrophotometer
6
7 (Perkin-Elmer, Norwalk, CT). Samples were excited at 496 nm, and emission was at 516 nm.
8
9

10 11 *2.6. Measurement of microtubular content*

12
13
14 The microtubular content was determined following a previously published procedure
15
16 [29]. Briefly, samples of cell suspensions (200 μ L; 2×10^6 cells/mL) were transferred to 200
17
18 μ L of ice-cold 3% (w/v) formaldehyde in PBS for 10 min. Fixed cells were permeabilized by
19
20 incubation for 10 min with 0.025% (v/v) Nonidet P40 detergent dissolved in PBS and then
21
22 incubated for 30 min with Oregon Green 488 paclitaxel (1 μ M) in PBS supplemented with
23
24 0.5% (w/v) BSA. Cells were collected by centrifugation and resuspended in PBS. Cell
25
26 staining was measured using a Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer,
27
28 Norwalk, CT). Samples were excited at 488 nm and emission was at 522 nm.
29
30
31
32
33

34 35 *2.7. Statistical Analysis*

36
37
38 Analysis of statistical significance was performed using one-way analysis of variance
39
40 combined with the Dunnett tests. The difference was considered statistically significant when
41
42 at least $p < 0.05$.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ indicative of SOCE (the integral of the TG-evoked rise in $[\text{Ca}^{2+}]_c$ for 3 min after addition of CaCl_2 was $15327 \pm 1221 \text{ nM}\cdot\text{s}$; 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 $75 \pm 9\%$ and $80 \pm 11\%$, respectively (Figs. 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 polymerisation after stimulation with 1 μM TG (Table 1; $p < 0.05$; $n = 6$). Cell treatment with colchicine enhanced TG-evoked SOCE by $23 \pm 4\%$ (the integral of the TG-evoked rise in $[\text{Ca}^{2+}]_c$ for 3 min after addition of CaCl_2 in cells pretreated with colchicine was $18975 \pm 543 \text{ nM}\cdot\text{s}$; $p < 0.05$), 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

1 association between STIM1 and both Orai1 and TRPC1. As shown in Figs. 1B and C, top
2 panels, treatment with colchicine enhanced TG-evoked association between STIM1 and
3 Orai1 by $75 \pm 9\%$ and between STIM1 and TRPC1 by $44 \pm 6\%$ ($p < 0.05$), without affecting
4 the association between these proteins in non-stimulated cells ($n=6$). Western blotting with
5 the immunoprecipitating antibody confirms that a similar amount of protein was loaded in all
6 lanes (Figs. 1B and C, bottom panels). Conversely, treatment of HEK-293 cells for 30 min
7 with 10 μM paclitaxel at 37 °C enhanced the microtubular content in non-stimulated cells
8 and prevented TG-evoked tubulin polymerisation (Table 1; $p < 0.05$; $n=6$). Treatment with
9 paclitaxel attenuated TG-evoked SOCE by $60 \pm 7\%$ (the integral of TG-evoked rise in
10 $[\text{Ca}^{2+}]_c$ for 3 min after addition of CaCl_2 in cells pretreated with paclitaxel was 6238 ± 1508
11 $\text{nM}\cdot\text{s}$; $p < 0.05$), without having any effect on TG-evoked Ca^{2+} efflux from the stores (Fig.
12 2A). In addition, pretreatment with paclitaxel reduced TG-induced association between
13 STIM1 and both Orai1 and TRPC1 by $49 \pm 6\%$ and $59 \pm 5\%$, respectively ($p < 0.05$), without
14 affecting the interaction between these proteins in non-stimulated cells (Figs. 2B and C, top
15 panels; $n=6$). Western blotting with the immunoprecipitating antibody confirms that a similar
16 amount of protein was loaded in all lanes (Figs. 2B and C, bottom panels).

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

47 Jasplakinolide, a cell-permeant peptide isolated from *Jaspis johnstoni*, induces
48 polymerization and stabilization of actin filaments underneath the plasma membrane [33];
49 therefore, it is a useful tool to explore the role of the cortical actin cytoskeleton in the
50 activation of SOCE and its relevance in the association between STIM1 and the Ca^{2+}
51 channels in the plasma membrane. Cell treatment for 30 min with 10 μM jasplakinolide at 37
52 °C resulted in a significant increase in the F-actin content in non-stimulated cells and

1 prevented TG-induced further actin filament polymerisation (Table 2; $p<0.05$; $n=6$).
2
3 Treatment with jasplakinolide attenuated SOCE by $67 \pm 7\%$ (the integral of TG-evoked rise
4
5 in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ in cells pretreated with jasplakinolide was $5118 \pm$
6
7 $1867 \text{ nM}\cdot\text{s}$; $p<0.05$), without having any effect on TG-evoked Ca^{2+} release from the
8
9 intracellular stores (Fig. 3A). In cells treated with jasplakinolide TG was unable to enhance
10
11 association between STIM1 and both Orai1 and TRPC1 (Figs. 3B and C, top panels;
12
13 $p<0.05$). Treatment with jasplakinolide did not affect the interaction between these proteins
14
15 in cells not treated with TG (Figs. 3B and C, top panels). Western blotting with the anti-
16
17 STIM1 antibody confirms a similar amount of protein loaded in all lanes (Figs. 3B and C,
18
19 bottom panels).
20
21
22
23
24
25

26 *3.3. Disruption of the actin cytoskeleton enhances TG-evoked SOCE*

27
28

29
30 In order to investigate the role of the actin filament network in SOCE and TG-evoked
31
32 association between STIM1 and the Ca^{2+} channels Orai1 and TRPC1, we used Cyt D, a
33
34 widely utilized cell-permeant inhibitor of actin polymerization [17, 34]. Pretreatment of
35
36 HEK-293 cells for 40 min at 37°C with $10 \mu\text{M}$ Cyt D reduced the F-actin content in non-
37
38 stimulated cells and impaired TG-induced F-actin polymerisation (Table 2; $p<0.05$; $n=6$). In
39
40 addition, treatment with Cyt D enhanced TG-evoked Ca^{2+} entry by $71 \pm 5\%$ (the integral of
41
42 the rise in $[Ca^{2+}]_c$ for 3 min after addition of $CaCl_2$ was $25190 \pm 1780 \text{ nM}\cdot\text{s}$ in Cyt D-treated
43
44 cells, Fig. 4A; $p<0.05$; $n=6$). In contrast to the effect of Cyt D on TG-evoked Ca^{2+} entry,
45
46
47 treatment of HEK-293 cells with $10 \mu\text{M}$ Cyt D for 40 min at 37°C did not significantly
48
49 modify the association between STIM1 and both Orai1 and TRPC1 in non-stimulated cells
50
51 or cells treated with TG (Fig. 4B and 4C, top panels). Western blotting with the
52
53 immunoprecipitating antibody confirms a similar amount of protein loaded in all lanes (Figs.
54
55 4B and C, bottom panels).
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3.4. Cytochalasin D impairs 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 $215 \pm 47\%$ and $121 \pm 27\%$, respectively (Figs. 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 (Figs. 5A and B, top panels; $p < 0.05$; $n = 6$). Western blotting with the immunoprecipitating antibody confirms a similar amount of protein loaded in all lanes (Figs. 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 $169 \pm 26\%$ and $45 \pm 10\%$, respectively, as detected by co-immunoprecipitation (Figs. 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 (Figs. 5C and D; $p < 0.05$; $n = 6$), thus suggesting that this association requires the integrity of

1 the actin filament network.

2
3
4 Previous studies have reported that calmodulin antagonists potentiate SOCE in pulmonary
5
6 artery smooth muscle cells [37], and cerebellar granule cells [38] and astrocytes [39]. Hence,
7
8 we have further investigated the effect of the calmodulin antagonist calmidazolium on TG-
9
10 evoked Ca^{2+} entry in HEK-293 cells. Our results indicate that treatment for 10 min with 1
11
12 μM calmidazolium enhances TG-induced calcium entry by $63 \pm 7\%$ (the integral of the rise
13
14 in $[\text{Ca}^{2+}]_c$ for 3 min after addition of CaCl_2 was $25092 \pm 356 \text{ nM}\cdot\text{s}$ in calmidazolium-treated
15
16 cells), without inducing significant changes in TG-evoked Ca^{2+} release (Fig. 6; $p < 0.05$; $n = 6$).
17
18
19 The effect of calmidazolium was similar to that observed when cells were pretreated with
20
21 calmidazolium in the presence of Cyt D, which enhanced TG-evoked SOCE by $69 \pm 5\%$ (the
22
23 integral of the rise in $[\text{Ca}^{2+}]_c$ for 3 min after addition of CaCl_2 was $26034 \pm 249 \text{ nM}\cdot\text{s}$ in
24
25 calmidazolium+Cyt D-treated cells; Fig. 6; $p < 0.05$; $n = 6$); thus indicating that the effect of
26
27 Cyt D enhancing TG-evoked Ca^{2+} entry might be attributed to inhibition of the regulatory
28
29 role of calmodulin on SOCE.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 signaling 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 DDT₁MF-2 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 and co-workers 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 three minutes after the addition of TG, while in the study published by Smyth and co-workers 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

1 significant effect on the association between STIM1 and Orai1 or STIM1 and TRPC1.
2
3 Interestingly, we have found that microfilament disruption results in inhibition of TG-
4
5 induced association between calmodulin and both Orai1 and TRPC1 (see Fig. 7). Since
6
7 inhibition of calmodulin results in a similar increase in TG-evoked SOCE than disruption of
8
9 the actin network our results provide evidence for a role of calmodulin in the regulation of
10
11 SOCE through its interaction with Orai1 and TRPC1 supported by the actin cytoskeleton.
12
13 The microtubules must play a further important regulatory role in the association of STIM1
14
15 with the Ca^{2+} channels as previously reported [12], since disruption of the microtubules
16
17 enhances this association, an event that has not been found after inhibition of actin
18
19 polymerization.
20
21
22
23

24
25 Calmodulin has been reported to interact with TRP proteins, including *Drosophila* TRPL
26
27 [52] and mammalian TRPC1 [35]. More recently, calmodulin was found to interact with the
28
29 polybasic C-termini of STIM1 [53] and with a membrane-proximal N-terminal domain of
30
31 Orai1 [36] in a Ca^{2+} dependent manner. Different experimental manoeuvres have
32
33 demonstrated that calmodulin is involved in Ca^{2+} -dependent inactivation of SOCE, including
34
35 expression of TRPC1 or Orai1 mutants lacking the ability to bind calmodulin, which
36
37 prevented Ca^{2+} -dependent inactivation of SOCE in human salivary gland cells [35], or I_{CRAC}
38
39 in HEK-293 cells [36], respectively, or over-expression of a calmodulin inhibitor peptide and
40
41 a Ca^{2+} -insensitive calmodulin mutant, which results in reduced inactivation of I_{SOC} in liver
42
43 cells [54]. Our results indicate that association of calmodulin with the Ca^{2+} channels Orai1
44
45 and TRPC1 requires the integrity of the actin cytoskeleton, which might provide a support
46
47 for these associations, and, therefore, disruption of the actin network leads to impairment of
48
49 Ca^{2+} -dependent inactivation of SOCE reported above.
50
51
52
53
54
55
56
57

58 We have not performed experiments to assess whether the effect of the cytoskeleton
59
60
61
62
63
64
65

1 modifiers is maximal; however, the changes induced in the microtubules and actin filaments
2
3 are sufficiently evident to demonstrate that the cytoskeleton plays a relevant role in the
4
5 regulation of SOCE in HEK-293 cells through the regulation of the interaction between
6
7 STIM1 and Orai1 or TRPC1. In addition, the actin cytoskeleton supports the association of
8
9 calmodulin with the Ca^{2+} channels in the plasma membrane, which provides an additional
10
11 regulatory role of store-operated Ca^{2+} influx.
12
13
14
15
16

17 *Acknowledgements*

18
19
20 Supported by MICINN (Grants BFU2010-21043-C02-01 and BFU2010-21043-C02-02)
21
22 and Junta de Extremadura- FEDER (GR10010). C.G. and N.D. are supported by Spanish
23
24 Ministry of Science and Innovation (PTA2008- 0870-P) and Junta de Extremadura
25
26 (PRE09020), respectively.
27
28
29
30
31
32

33 *Disclosure of conflict of interests*

34
35
36 The authors state that there is no conflict of interest.
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References

- 1
2
3 [1] Putney JW, Bird GS. Cytoplasmic calcium oscillations and store-operated calcium
4 influx. *J Physiol* 2008;586:3055-9.
5
6 [2] Stathopoulos PB, Zheng L, Li GY, Plevin MJ, Ikura M. Structural and mechanistic
7 insights into STIM1-mediated initiation of store-operated calcium entry. *Cell*
8 2008;135:110-22.
9
10 [3] Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, et al. STIM1 is a
11 Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the
12 plasma membrane. *Nature* 2005;437:902-5.
13 [4] Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S. SOAR and the
14 polybasic STIM1 domains gate and regulate Orai channels. *Nat Cell Biol*
15 2009;11:337-43.
16 [5] Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, Raunser S, et al.
17 STIM1 clusters and activates CRAC channels via direct binding of a cytosolic
18 domain to Orai1. *Cell* 2009;136:876-90.
19 [6] Muik M, Fahrner M, Derler I, Schindl R, Bergsmann J, Frischauf I, et al. A Cytosolic
20 Homomerization and a Modulatory Domain within STIM1 C Terminus Determine
21 Coupling to ORAI1 Channels. *J Biol Chem* 2009;284:8421-6.
22 [7] Derler I, Fahrner M, Muik M, Lackner B, Schindl R, Groschner K, et al. A CRAC
23 modulatory domain (CMD) within STIM1 mediates fast Ca^{2+} -dependent inactivation
24 of ORAI1 channels. *J Biol Chem* 2009.
25 [8] Yu F, Sun L, Courjaret R, Machaca K. Role of the STIM1 C-terminal domain in
26 STIM1 clustering. *J Biol Chem* 2011;doi/10.1074/jbc.M110.188789.
27 [9] Muik M, Frischauf I, Derler I, Fahrner M, Bergsmann J, Eder P, et al. Dynamic
28 coupling of the putative coiled-coil domain of ORAI1 with STIM1 mediates ORAI1
29 channel activation. *J Biol Chem* 2008;283:8014-22.
30 [10] Wang Y, Deng X, Gill DL. Calcium signaling by STIM and Orai: intimate coupling
31 details revealed. *Sci Signal* 2010;3:pe42.
32 [11] Zeng W, Yuan JP, Kim MS, Choi YJ, Huang GN, Worley PF, et al. STIM1 gates
33 TRPC channels, but not Orai1, by electrostatic interaction. *Mol Cell* 2008;32:439-48.
34 [12] Smyth JT, DeHaven WI, Bird GS, Putney JW, Jr. Role of the microtubule
35 cytoskeleton in the function of the store-operated Ca^{2+} channel activator STIM1. *J*
36 *Cell Sci* 2007;120:3762-71.
37 [13] Ribeiro CM, Reece J, Putney JW, Jr. Role of the cytoskeleton in calcium signaling in
38 NIH 3T3 cells. An intact cytoskeleton is required for agonist-induced $[Ca^{2+}]_i$
39 signaling, but not for capacitative calcium entry. *J Biol Chem* 1997;272:26555-61.
40 [14] Patterson RL, van Rossum DB, Gill DL. Store-operated Ca^{2+} entry: evidence for a
41 secretion-like coupling model. *Cell* 1999;98:487-99.
42 [15] Bakowski D, Glitsch MD, Parekh AB. An examination of the secretion-like coupling
43 model for the activation of the Ca^{2+} release-activated Ca^{2+} current I_{CRAC} in
44 RBL-1 cells. *J Physiol* 2001;532:55-71.
45 [16] Baba Y, Hayashi K, Fujii Y, Mizushima A, Watarai H, Wakamori M, et al. Coupling
46 of STIM1 to store-operated Ca^{2+} entry through its constitutive and inducible
47 movement in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 2006;103:16704-
48 9.
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [17] Rosado JA, Jenner S, Sage SO. A role for the actin cytoskeleton in the initiation and
2 maintenance of store-mediated calcium entry in human platelets. Evidence for
3 conformational coupling. *J Biol Chem* 2000;275:7527-33.
- 4 [18] Rosado JA, Lopez JJ, Harper AG, Harper MT, Redondo PC, Pariente JA, et al. Two
5 pathways for store-mediated calcium entry differentially dependent on the actin
6 cytoskeleton in human platelets. *J Biol Chem* 2004;279:29231-5.
- 7 [19] Redondo PC, Harper AG, Sage SO, Rosado JA. Dual role of tubulin-cytoskeleton in
8 store-operated calcium entry in human platelets. *Cell Signal* 2007;19:2147-54.
- 9 [20] Holda JR, Blatter LA. Capacitative calcium entry is inhibited in vascular endothelial
10 cells by disruption of cytoskeletal microfilaments. *FEBS Lett* 1997;403:191-6.
- 11 [21] Redondo PC, Lajas AI, Salido GM, Gonzalez A, Rosado JA, Pariente JA. Evidence
12 for secretion-like coupling involving pp60src in the activation and maintenance of
13 store-mediated Ca²⁺ entry in mouse pancreatic acinar cells. *Biochem J*
14 2003;370:255-63.
- 15 [22] Redondo PC, Harper MT, Rosado JA, Sage SO. A role for cofilin in the activation of
16 store-operated calcium entry by de novo conformational coupling in human platelets.
17 *Blood* 2006;107:973-9.
- 18 [23] Redondo PC, Ben-Amor N, Salido GM, Bartegi A, Pariente JA, Rosado JA. Ca²⁺-
19 independent activation of Bruton's tyrosine kinase is required for store-mediated
20 Ca²⁺ entry in human platelets. *Cell Signal* 2005;17:1011-21.
- 21 [24] Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC. The Ca²⁺-
22 mobilizing potency of alpha-thrombin and thrombin-receptor-activating peptide on
23 human platelets -- concentration and time effects of thrombin-induced Ca²⁺-
24 signaling. *Eur J Biochem* 1997;249:547-55.
- 25 [25] Jardin I, Ben Amor N, Bartegi A, Pariente JA, Salido GM, Rosado JA. Differential
26 involvement of thrombin receptors in Ca²⁺ release from two different intracellular
27 stores in human platelets. *Biochem J* 2007;401:167-74.
- 28 [26] Jardin I, Redondo PC, Salido GM, Rosado JA. Phosphatidylinositol 4,5-bisphosphate
29 enhances store-operated calcium entry through hTRPC6 channel in human platelets.
30 *Biochim Biophys Acta* 2008;1783:84-97.
- 31 [27] Woodard GE, Lopez JJ, Jardin I, Salido GM, Rosado JA. TRPC3 regulates agonist-
32 stimulated Ca²⁺ mobilization by mediating the interaction between type I inositol
33 1,4,5-trisphosphate receptor, RACK1, and Orai1. *J Biol Chem* 2010;285:8045-53.
- 34 [28] De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, et al.
35 Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate
36 receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J*
37 *Biol Chem* 2007;282:11590-601.
- 38 [29] Bouaziz A, Amor NB, Woodard GE, Zibidi H, Lopez JJ, Bartegi A, et al. Tyrosine
39 phosphorylation / dephosphorylation balance is involved in thrombin-evoked
40 microtubular reorganisation in human platelets. *Thromb Haemost* 2007;98:375-84.
- 41 [30] Canizares C, Vivar N, Herdoiza M. Role of the microtubular system in platelet
42 aggregation. *Braz J Med Biol Res* 1994;27:1533-51.
- 43 [31] White JG, Krumwiede M, Sauk JJ. Microtubule reassembly in surface-activated
44 platelets. *Blood* 1985;65:1494-503.
- 45 [32] Parekh H, Simpkins H. The transport and binding of taxol. *Gen Pharmacol*
46 1997;29:167-72.
- 47 [33] Rosado JA, Sage SO. The actin cytoskeleton in store-mediated calcium entry. *J*
48 *Physiol* 2000;526 Pt 2:221-9.
- 49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [34] Rosado JA, Sage SO. Farnesylcysteine analogues inhibit store-regulated Ca²⁺ entry
2 in human platelets: evidence for involvement of small GTP-binding proteins and
3 actin cytoskeleton. *Biochem J* 2000;347 Pt 1:183-92.
- 4 [35] Singh BB, Liu X, Tang J, Zhu MX, Ambudkar IS. Calmodulin regulates Ca(2+)-
5 dependent feedback inhibition of store-operated Ca(2+) influx by interaction with a
6 site in the C terminus of TrpC1. *Mol Cell* 2002;9:739-50.
- 7 [36] Mullins FM, Park CY, Dolmetsch RE, Lewis RS. STIM1 and calmodulin interact
8 with Orai1 to induce Ca²⁺-dependent inactivation of CRAC channels. *Proc Natl*
9 *Acad Sci U S A* 2009;106:15495-500.
- 10 [37] McElroy SP, Drummond RM, Gurney AM. Regulation of store-operated Ca²⁺ entry
11 in pulmonary artery smooth muscle cells. *Cell Calcium* 2009;46:99-106.
- 12 [38] Singaravelu K, Lohr C, Deitmer JW. Calcium-independent phospholipase A2
13 mediates store-operated calcium entry in rat cerebellar granule cells. *Cerebellum*
14 2008;7:467-81.
- 15 [39] Singaravelu K, Lohr C, Deitmer JW. Regulation of store-operated calcium entry by
16 calcium-independent phospholipase A2 in rat cerebellar astrocytes. *J Neurosci*
17 2006;26:9579-92.
- 18 [40] Smyth JT, Dehaven WI, Bird GS, Putney JW, Jr. Ca²⁺-store-dependent and -
19 independent reversal of Stim1 localization and function. *J Cell Sci* 2008;121:762-72.
- 20 [41] Itagaki K, Kannan KB, Singh BB, Hauser CJ. Cytoskeletal reorganization internalizes
21 multiple transient receptor potential channels and blocks calcium entry into human
22 neutrophils. *J Immunol* 2004;172:601-7.
- 23 [42] Korkiamaki T, Yla-Outinen H, Koivunen J, Peltonen J. An intact actin-containing
24 cytoskeleton is required for capacitative calcium entry, but not for ATP-induced
25 calcium-mediated cell signaling in cultured human keratinocytes. *Med Sci Monit*
26 2003;9:BR199-207.
- 27 [43] Kawamura M, Terasaka O, Ebisawa T, Kondo I, Masaki E, Ahmed A, et al. Integrity
28 of actin-network is involved in uridine 5'-triphosphate evoked store-operated Ca²⁺
29 entry in bovine adrenocortical fasciculata cells. *J Pharmacol Sci* 2003;91:23-33.
- 30 [44] Sabala P, Targos B, Caravelli A, Czajkowski R, Lim D, Gragnaniello G, et al. Role
31 of the actin cytoskeleton in store-mediated calcium entry in glioma C6 cells. *Biochem*
32 *Biophys Res Commun* 2002;296:484-91.
- 33 [45] Harper AG, Sage SO. A key role for reverse Na⁺/Ca²⁺ exchange influenced by the
34 actin cytoskeleton in store-operated Ca²⁺ entry in human platelets: evidence against
35 the de novo conformational coupling hypothesis. *Cell Calcium* 2007;42:606-17.
- 36 [46] Camello C, Pariente JA, Salido GM, Camello PJ. Sequential activation of different
37 Ca²⁺ entry pathways upon cholinergic stimulation in mouse pancreatic acinar cells. *J*
38 *Physiol* 1999;516 (Pt 2):399-408.
- 39 [47] Jenner S, Sage SO. Two pathways for store-mediated calcium entry in human
40 platelets. *Platelets* 2000;11:215-21.
- 41 [48] Jardin I, Lopez JJ, Salido GM, Rosado JA. Functional relevance of the de novo
42 coupling between hTRPC1 and type II IP₃ receptor in store-operated Ca²⁺ entry in
43 human platelets. *Cell Signal* 2008;20:737-47.
- 44 [49] Grigoriev I, Gouveia SM, van der Vaart B, Demmers J, Smyth JT, Honnappa S, et al.
45 STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. *Curr*
46 *Biol* 2008;18:177-82.
- 47 [50] Rosado JA, Brownlow SL, Sage SO. Endogenously expressed Trp1 is involved in
48 store-mediated Ca²⁺ entry by conformational coupling in human platelets. *J Biol*
49 *Chem* 2002;277:42157-63.
- 50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [51] Rosado JA, Sage SO. Activation of store-mediated calcium entry by secretion-like
2 coupling between the inositol 1,4,5-trisphosphate receptor type II and human
3 transient receptor potential (hTrp1) channels in human platelets. *Biochem J*
4 2001;356:191-8.
- 5 [52] Phillips AM, Bull A, Kelly LE. Identification of a *Drosophila* gene encoding a
6 calmodulin-binding protein with homology to the trp phototransduction gene. *Neuron*
7 1992;8:631-42.
- 8 [53] Bauer MC, O'Connell D, Cahill DJ, Linse S. Calmodulin binding to the polybasic C-
9 termini of STIM proteins involved in store-operated calcium entry. *Biochemistry*
10 2008;47:6089-91.
- 11 [54] Litjens T, Harland ML, Roberts ML, Barritt GJ, Rychkov GY. Fast Ca²⁺-dependent
12 inactivation of the store-operated Ca²⁺ current (ISOC) in liver cells: a role for
13 calmodulin. *J Physiol* 2004;558:85-97.
- 14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 μ M colchicine or 10 μ M paclitaxel for 30 min or the vehicles for the same period as controls. Cells were suspended in a Ca²⁺-free medium (1.2 mM EGTA added) and then treated with 1 μ M 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 mean \pm 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.

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 Ca²⁺-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 mean ± 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.

Figure legends

1
2
3 **Fig. 1.** Effect of colchicine on TG-evoked Ca^{2+} entry and association between STIM1 and
4 both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6
5 cells/mL) were incubated for 30 min in the absence or presence of 30 μM colchicine. At the
6 time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3
7 min later CaCl_2 (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate
8 Ca^{2+} entry in the presence or absence of colchicine, as indicated. Ca^{2+} entry was determined
9 as described in the materials and methods section. Values are means \pm S.E.M. of six
10 independent experiments; * $p < 0.05$. (B and C) HEK-293 cells were preincubated in presence
11 of 30 μM colchicine or the vehicle (Control), as indicated, suspended in a Ca^{2+} -free medium
12 (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates
13 were incubated overnight with 2 $\mu\text{g/mL}$ anti-STIM1 antibody and 25 $\mu\text{g/mL}$ protein-A
14 agarose and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent
15 Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes
16 were reprobated with the anti-STIM1 antibody for protein loading control (bottom panels). The
17 panels show results from one experiment representative of 5 others. Molecular masses
18 indicated on the right were determined using molecular-mass markers run in the same gel.
19 Histograms represent the quantification of STIM1-Orai1 or STIM1-TRPC1 association in
20 non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical
21 density units and expressed as means \pm S.E.M. * $p < 0.05$, n.s., non significant. PC and PFC,
22 primary control and primary-free control, respectively. For PC, whole cell lysates were
23 subjected to 10% SDS/PAGE and subsequent Western blotting with the corresponding
24 primary and secondary antibodies. For PFC, whole cell lysates were subjected to 10%

1 SDS/PAGE and the membrane was subsequently incubated solely with the secondary
2 antibody. HC, heavy chain of the immunoglobulin used for immunoprecipitation.
3
4
5
6

7 **Fig. 2.** Effect of paclitaxel on TG-evoked Ca^{2+} entry and association between STIM1 and
8 both Orai1 and TRPC1 in HEK-293 cells. (A) Fura-2-loaded HEK-293 cells (2×10^6
9 cells/mL) were incubated for 30 min in the absence or presence of 10 μM paclitaxel. At the
10 time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3
11 min later CaCl_2 (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate
12 Ca^{2+} entry in the presence or absence of paclitaxel, as indicated. Ca^{2+} entry was determined
13 as described in the materials and methods section. Values are means \pm S.E.M. of six
14 independent experiments; * $p < 0.05$. (B and C) HEK-293 cells were preincubated in presence
15 of 10 μM paclitaxel or the vehicle (Control), as indicated, suspended in a Ca^{2+} -free medium
16 (1.2 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates
17 were incubated overnight with 2 $\mu\text{g/mL}$ anti-STIM1 antibody and 25 $\mu\text{g/mL}$ protein-A
18 agarose and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent
19 Western blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes
20 were reprobbed with the anti-STIM1 antibody for protein loading control (bottom panels). The
21 panels show results from one experiment representative of 5 others. Molecular masses
22 indicated on the right were determined using molecular-mass markers run in the same gel.
23 Histograms represent the quantification of STIM1-Orai1 or STIM1-TRPC1 association in
24 non-stimulated (control) and TG-treated cells. Results are presented as arbitrary optical
25 density units and expressed as means \pm S.E.M. * $p < 0.05$, n.s., non significant. HC, heavy
26 chain of the immunoglobulin used for immunoprecipitation.
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 the materials and methods section. Values are means \pm 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 $\mu\text{g/mL}$ anti-STIM1 antibody and 25 $\mu\text{g/mL}$ 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 re probed 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 \pm 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

1 later CaCl₂ (2 mM) was added to the medium to initiate Ca²⁺ entry. Histograms indicate Ca²⁺
2 entry in the presence or absence of cytochalasin D, as indicated. Ca²⁺ entry was determined
3 as described in the materials and methods section. Values are means ± S.E.M. of six
4 independent experiments; * *p*<0.05. (B and C) HEK-293 cells were preincubated in presence
5 of 10 μM Cyt D or the vehicle (Control), as indicated, suspended in a Ca²⁺-free medium (1.2
6 mM EGTA added), stimulated for 3 min with 1 μM TG and lysed. Whole cell lysates were
7 incubated overnight with 2 μg/mL anti-STIM1 antibody and 25 μg/mL protein-A agarose
8 and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western
9 blotting with a specific anti-Orai1 (B) or anti-TRPC1 (C) antibody. Membranes were
10 reprobbed with the anti-STIM1 antibody for protein loading control. The panels show results
11 from one experiment representative of 5 others. Molecular masses indicated on the right
12 were determined using molecular-mass markers run in the same gel. Histograms represent
13 the quantification of STIM1-Orai1 or STIM1-TRPC1 association in non-stimulated (control)
14 and TG-treated cells. Results are presented as arbitrary optical density units and expressed as
15 means ± S.E.M. n.s., non significant. HC, heavy chain of the immunoglobulin used for
16 immunoprecipitation.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44

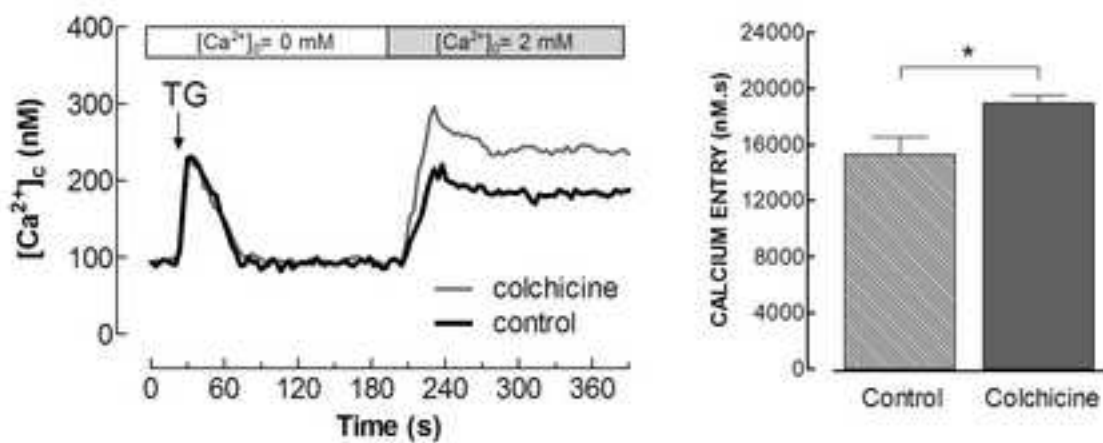
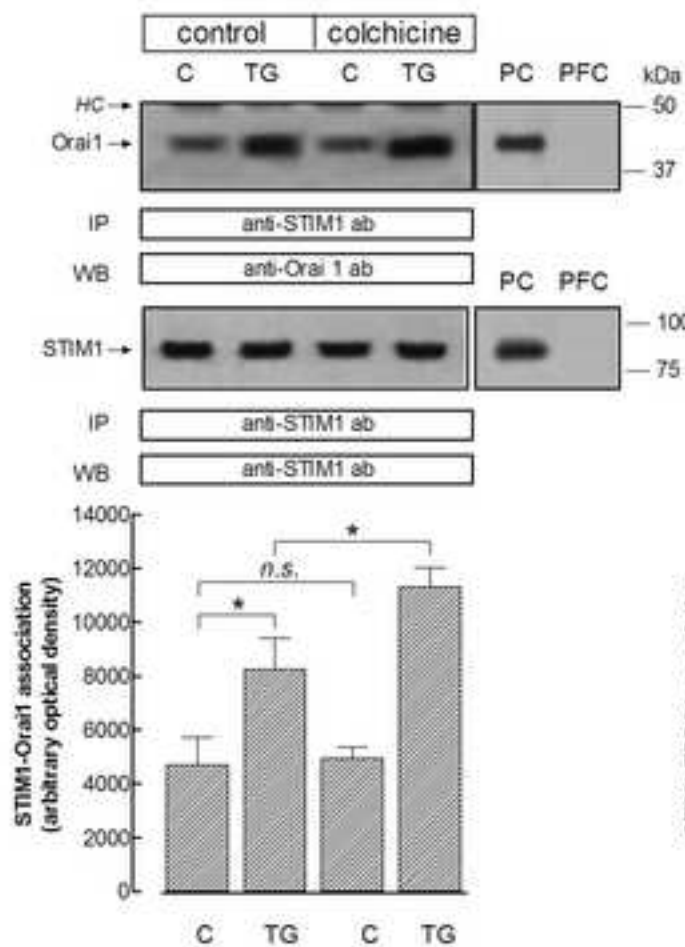
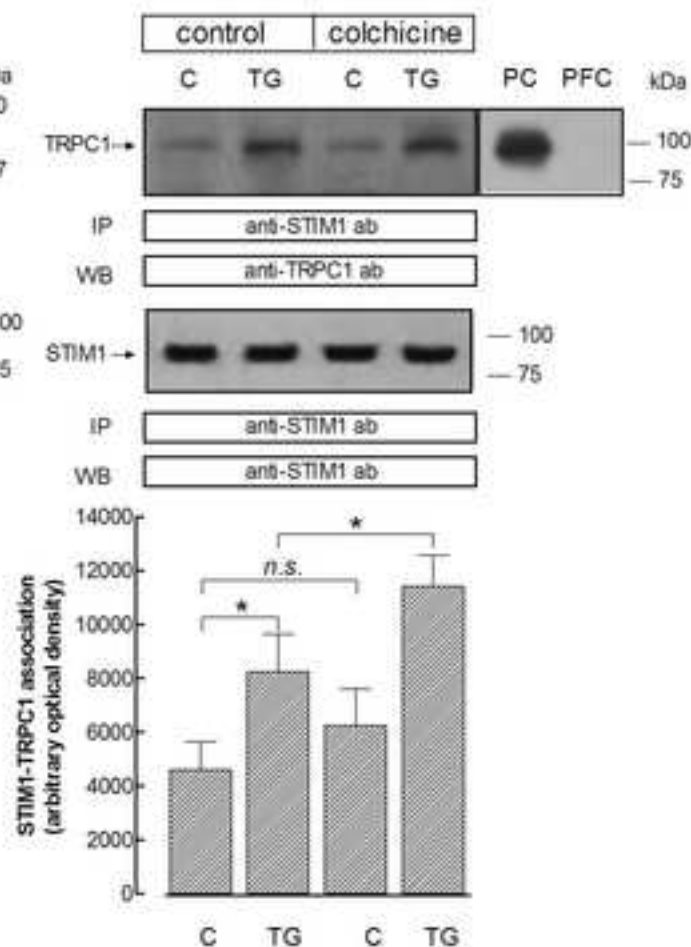
45 **Fig. 5.** Effect of cytochalasin D on TG-evoked association between calmodulin and both
46 Orai1 and TRPC1 in HEK-293 cells. HEK-293 cells (2 × 10⁶ cells/mL) were preincubated in
47 presence of 10 μM Cyt D or the vehicle (Control), as indicated, and either suspended in a
48 medium containing 1 mM Ca²⁺ (A and B) or suspended in a Ca²⁺-free medium (1.2 mM
49 EGTA added, C and D). Cells were then stimulated for 3 min with 1 μM TG and lysed.
50 Whole cell lysates were incubated overnight with 2 μg/mL anti-calmodulin antibody and 25
51 μg/mL protein-A agarose and immunoprecipitates were subjected to 10 % SDS-PAGE and
52
53
54
55
56
57
58
59
60
61
62
63
64
65

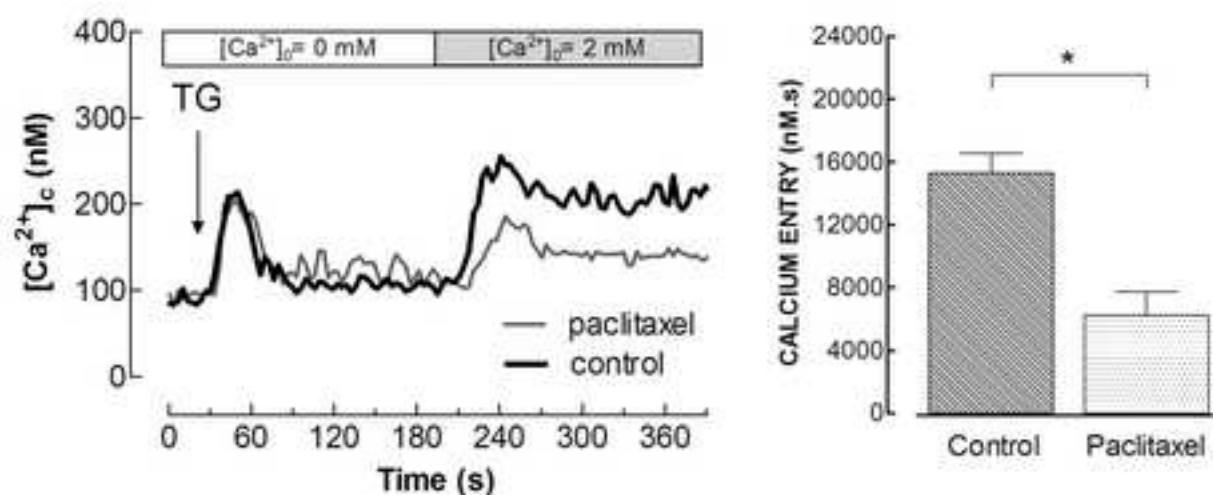
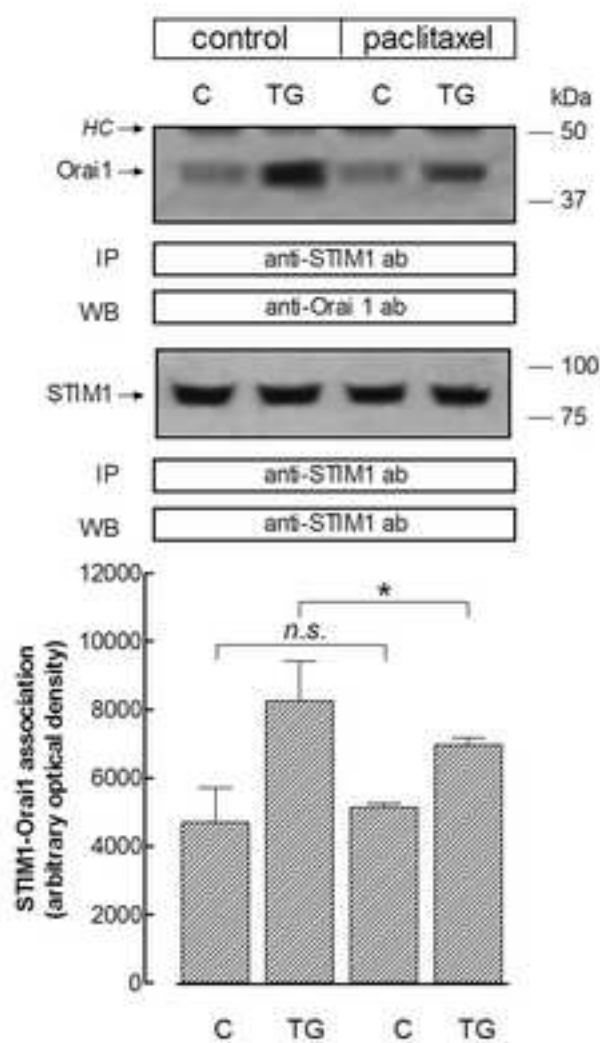
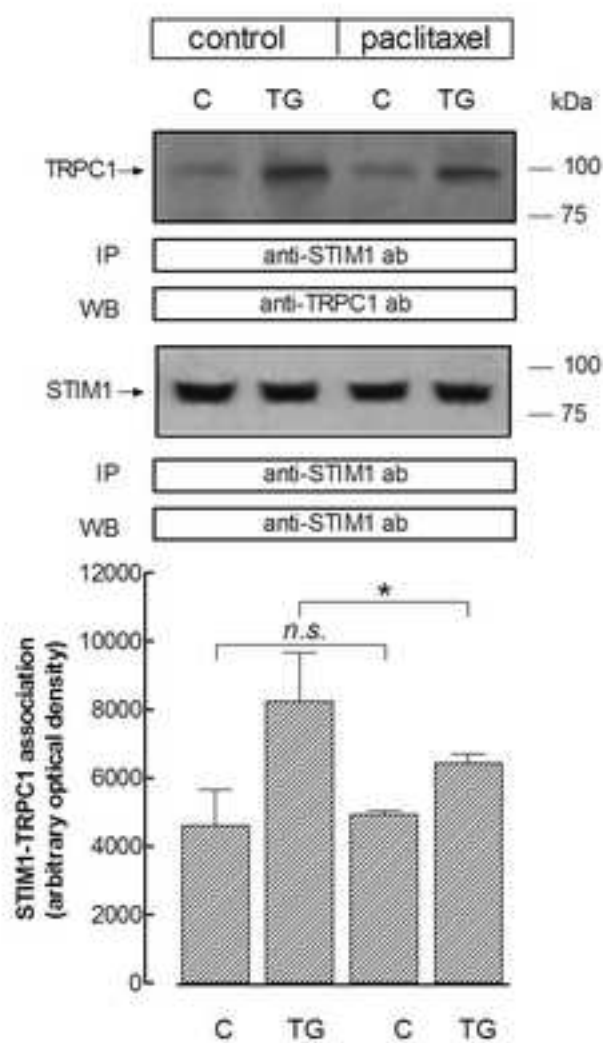
1 subsequent Western blotting with a specific anti-Orai1 or anti-TRPC1 antibody, as indicated.
2
3 Membranes were reprobbed with the anti-STIM1 antibody for protein loading control (bottom
4
5 panels). The panels show results from one experiment representative of 5 others. Molecular
6
7 masses indicated on the right were determined using molecular-mass markers run in the same
8
9 gel. Histograms represent the quantification of calmodulin-Orai1 or calmodulin-TRPC1
10
11 association in non-stimulated (control) and TG-treated cells. Results are presented as
12
13 percentage of control and expressed as means \pm S.E.M. * p <0.05, n.s., non significant. HC,
14
15 heavy chain of the immunoglobulin used for immunoprecipitation. PC and PFC, primary
16
17 control and primary-free control, respectively. For PC, whole cell lysates were subjected to
18
19 10% SDS/PAGE and subsequent Western blotting with the corresponding primary and
20
21 secondary antibodies. For PFC, whole cell lysates were subjected to 10% SDS/PAGE and the
22
23 membrane was subsequently incubated solely with the secondary antibody.
24
25
26
27
28
29
30
31

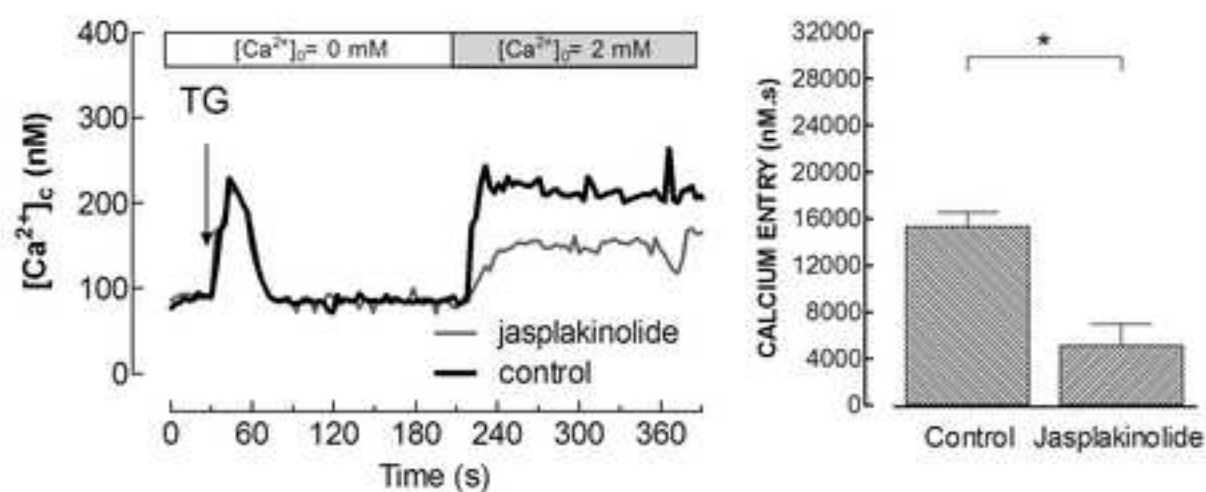
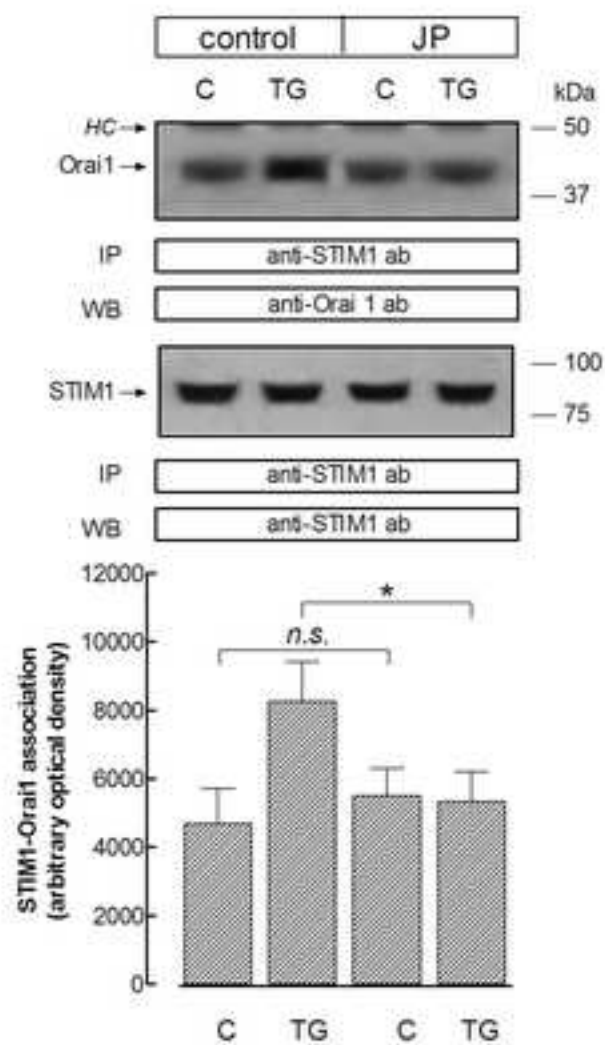
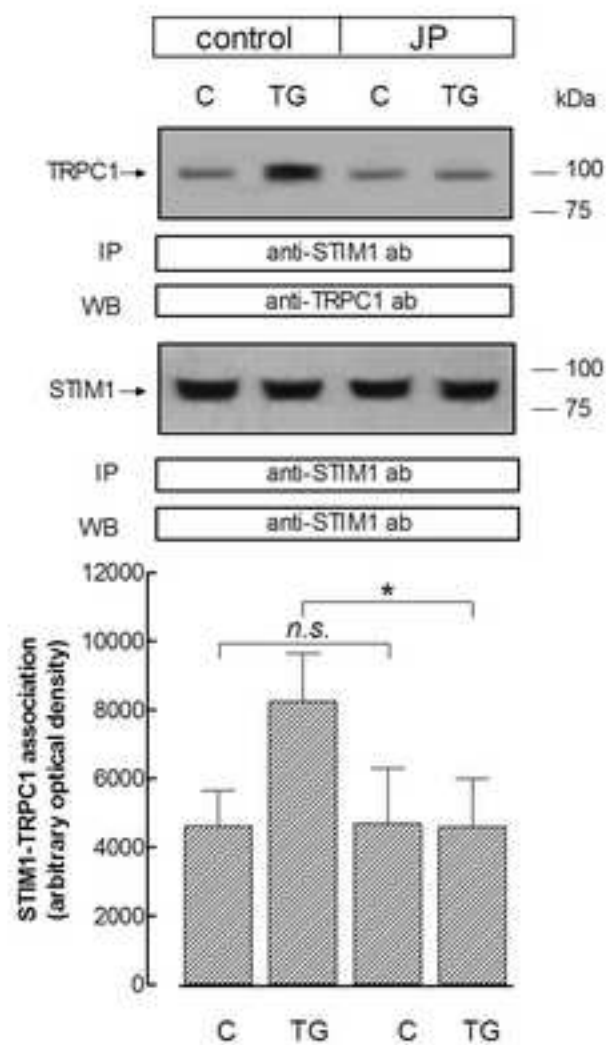
32 **Fig. 6.** Effect of calmidazolium and/or cytochalasin D on TG-evoked Ca^{2+} entry in HEK-293
33
34 cells. Fura-2-loaded HEK-293 cells (2×10^6 cells/mL) were incubated either for 40 min with
35
36 10 μM Cyt D, for 10 min with 1 μM calmidazolium, both or the vehicle as control. At the
37
38 time of experiment 1.2 mM EGTA was added. Cells were stimulated with 1 μM TG and 3
39
40 min later CaCl_2 (2 mM) was added to the medium to initiate Ca^{2+} entry. Histograms indicate
41
42 Ca^{2+} entry in the presence or absence of Cyt D, calmidazolium or both, as indicated. Ca^{2+}
43
44 entry was determined as described in materials and methods. Values are means \pm S.E.M. of
45
46 six independent experiments; * p <0.05. n.s., non significant.
47
48
49
50
51
52
53
54

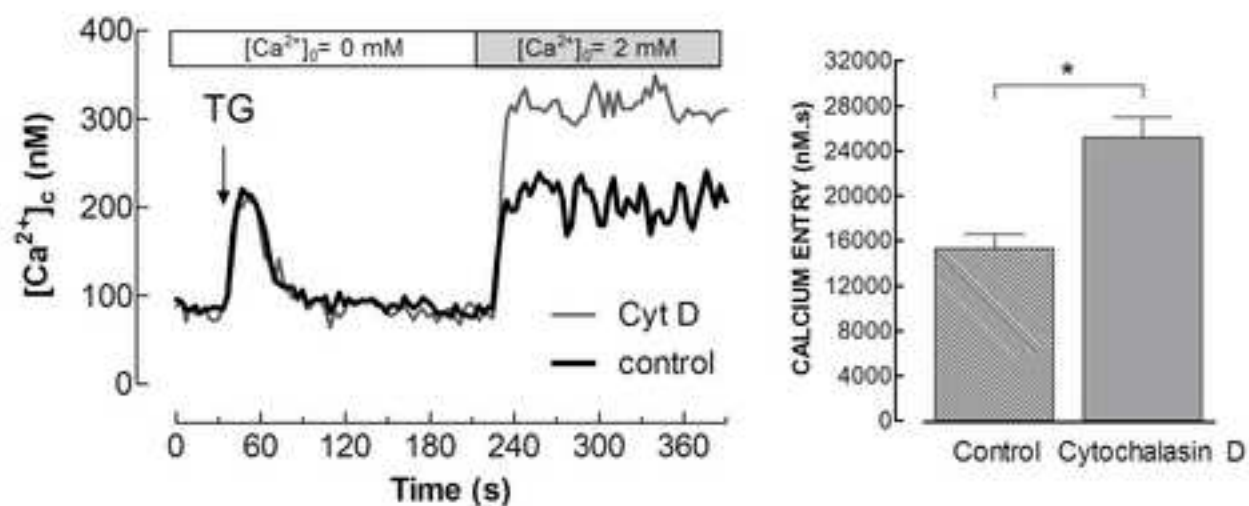
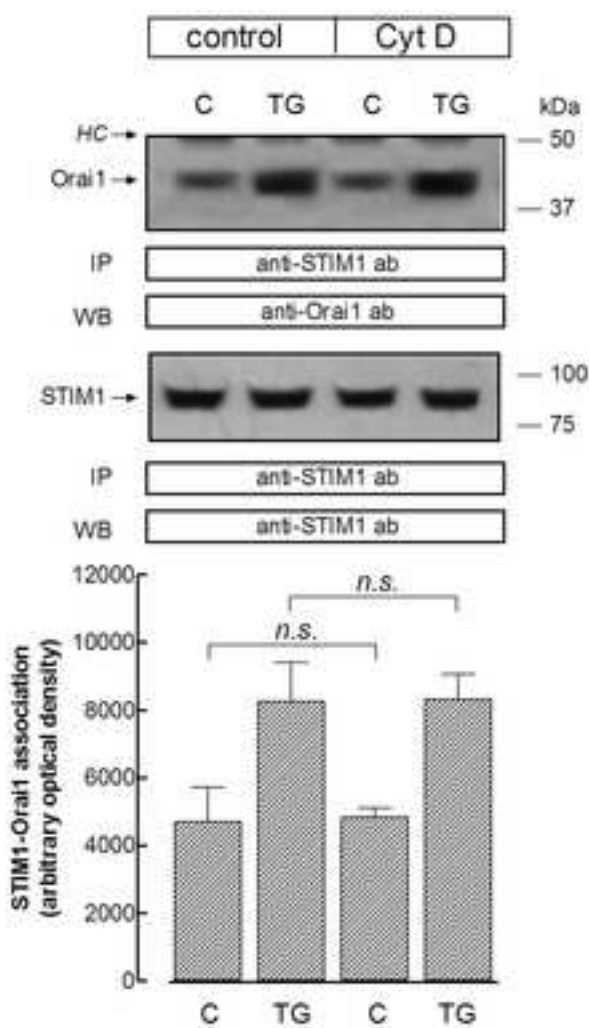
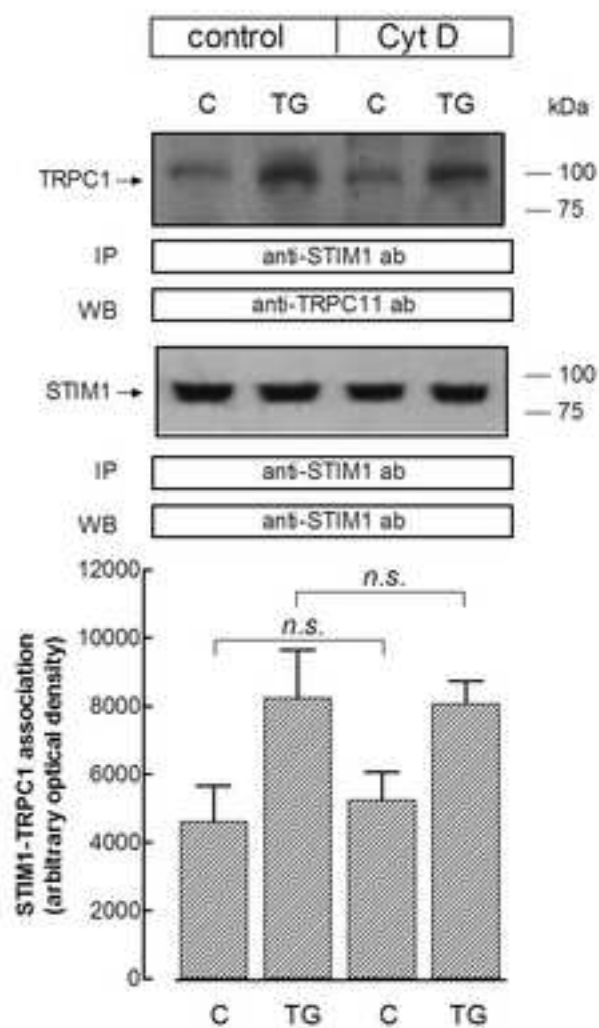
55 **Fig. 7.** Proposed model for cytoskeletal remodelling during SOCE in HEK-293 cells. A, in
56
57 the resting state the Ca^{2+} stores are full and the actin filaments and microtubules, located
58
59 beneath the plasma membrane, act as a cortical barrier preventing constitutive association
60
61
62
63
64
65

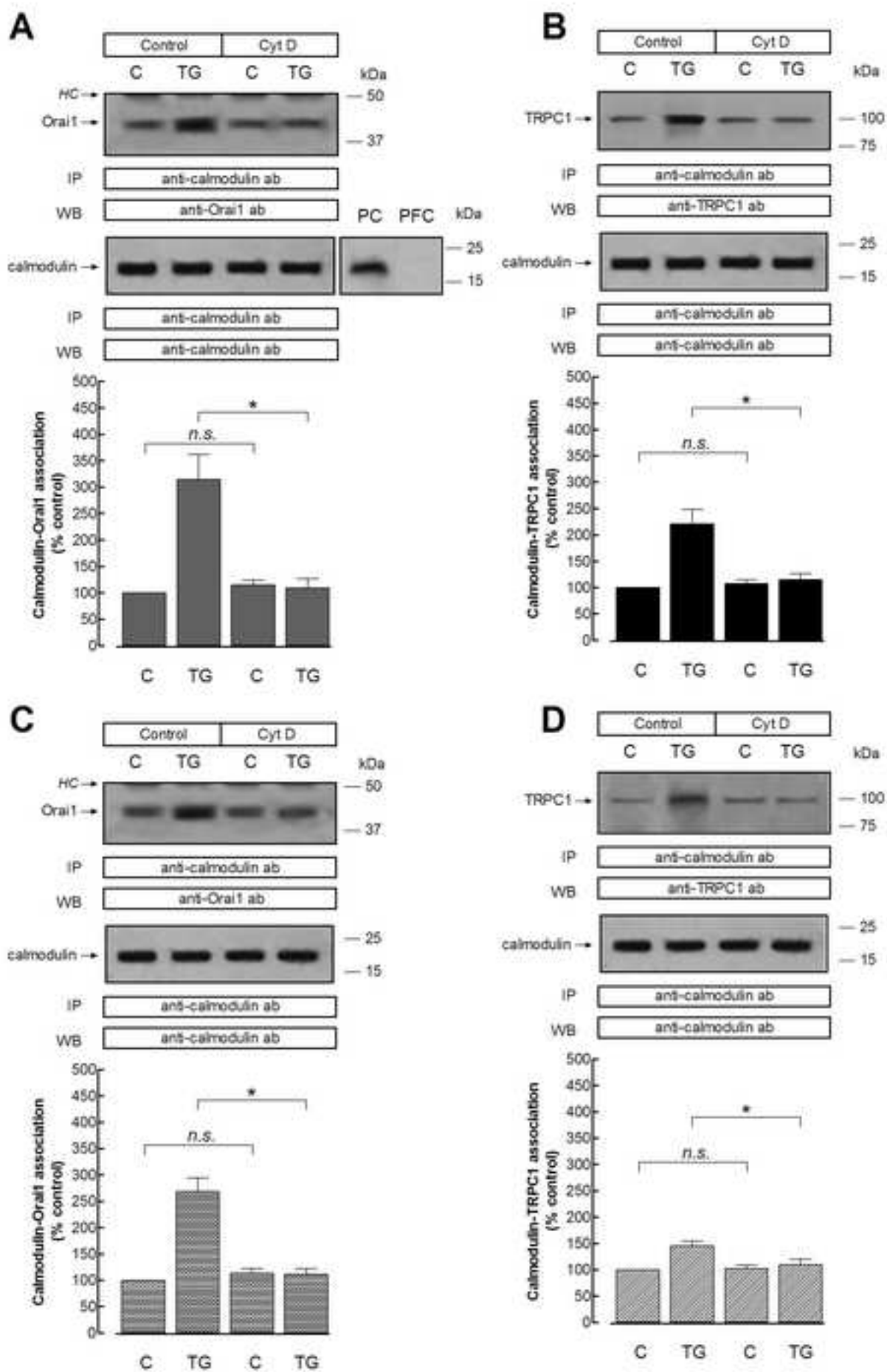
1 between STIM1 and SOC channels. B, Cell stimulation with physiological agonists (or
2 inhibition of Ca^{2+} reuptake by SERCA using thapsigargin (TG)) results in discharge of the
3 Ca^{2+} stores leading to remodelling of the actin and microtubular networks to facilitate the
4 association of STIM1 with SOC channels and the activation of SOCE. C and D, stabilization
5 of the actin or microtubular peripheral network prevents the association of STIM1 with SOC
6 channels and SOCE. E, Disruption of the microtubular barrier facilitates agonist (or store-
7 depletion)-induced association events and SOCE. F, disruption of the actin filament network
8 enhances agonist-stimulated SOCE by impairing the association of calmodulin with SOC
9 channels. ER, endoplasmic reticulum; G, heterotrimeric G protein; IP_3 , inositol 3,4,5-
10 trisphosphate; IP_3R , IP_3 receptor; PLC, phospholipase C; PM, plasma membrane; SOC,
11 store-operated Ca^{2+} channels (i.e. Orai1 and TRPC1),
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

A**B****C**

A**B****C**

A**B****C**

A**B****C**



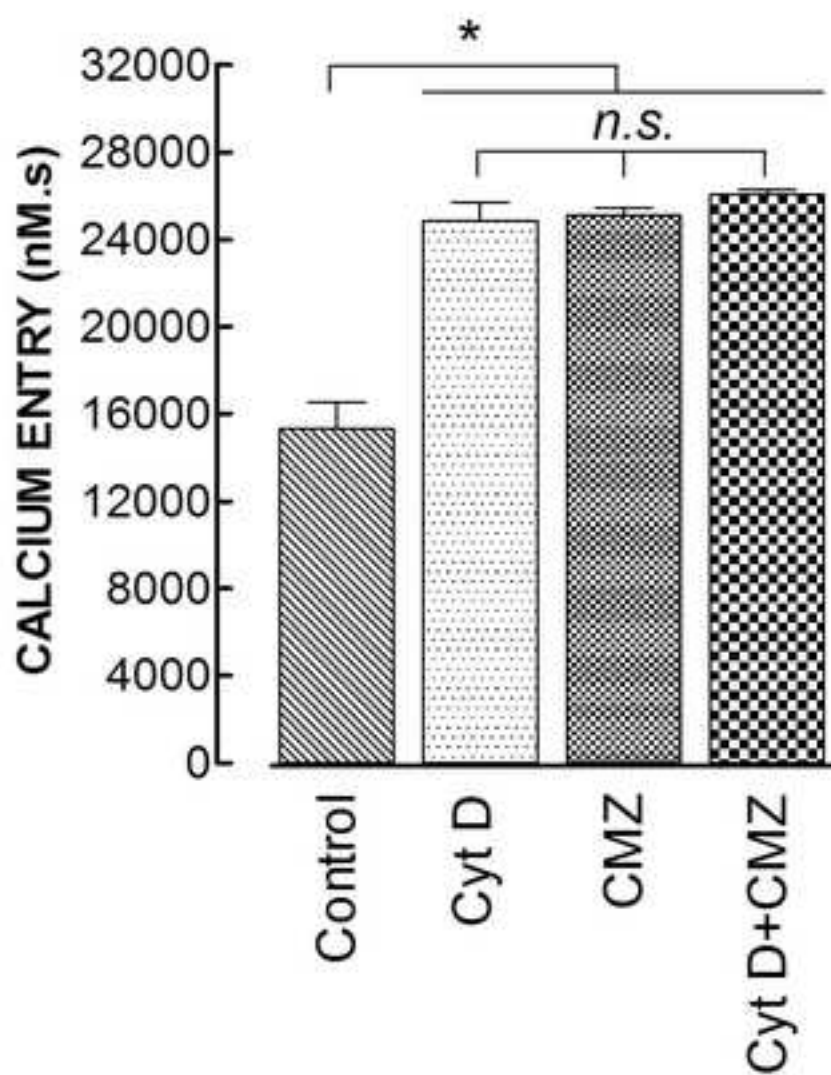
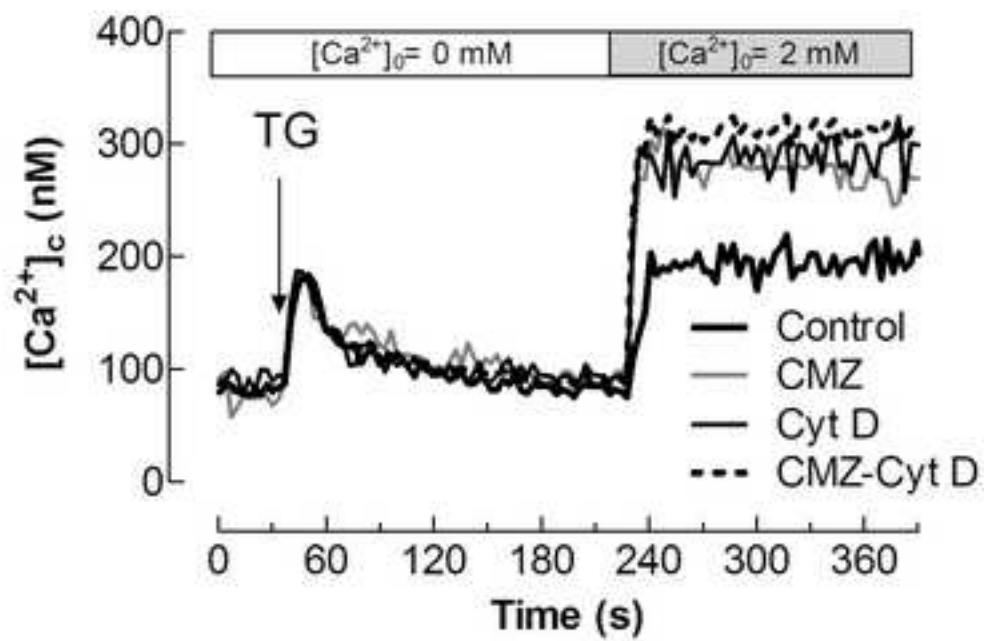
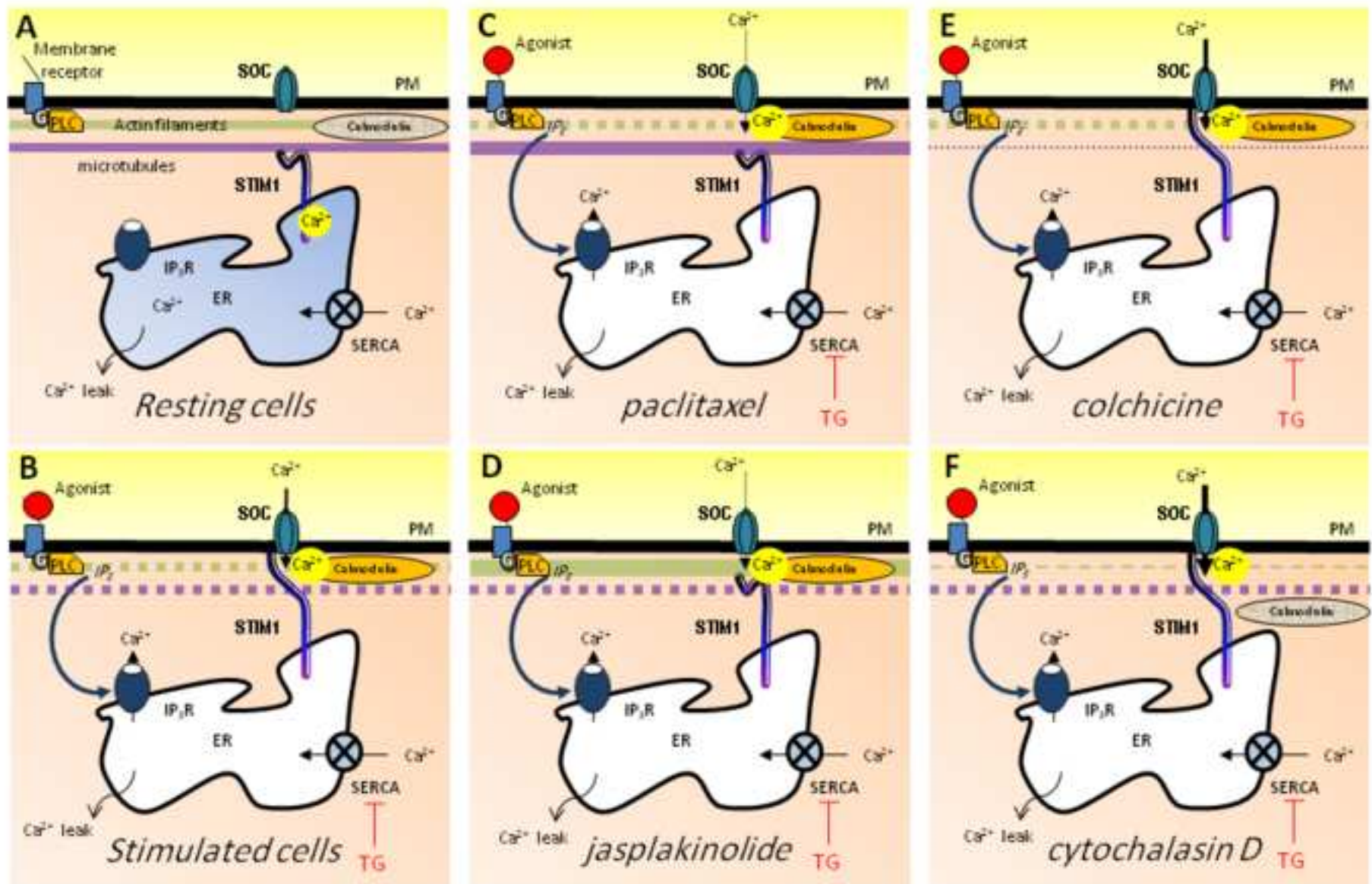


Figure 7



Manuscript

