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Two-pore channel 2 (TPC2) modulates store-operated Ca^{2+} entry

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ABSTRACT

Two-pore channels (TPCs) are NAADP-sensitive receptor channels that conduct Ca^{2+} efflux from the intracellular stores. Discharge of the internal Ca^{2+} pools results in the activation of store-operated Ca^{2+} entry (SOCE); however, the role of TPCs in the modulation of SOCE remains unexplored. Mammalian cells express three TPCs: TPC1, TPC2 and TPC3, a pseudogene in humans. Here we report that MEG01 and HEK293 cells endogenously express TPC1 and TPC2. Silencing TPC2 expression results in attenuation of the rate and extent of thapsigargin (TG)-evoked SOCE both in MEG01 and HEK293 cells, without having any effect on the ability of cells to accumulate Ca^{2+} into the TG-sensitive stores. Similarly, silencing of native TPC2 expression reduced thrombin-induced Ca^{2+} entry in MEG01 cells. In contrast, silencing of TPC1 expression was without effect either on TG or thrombin-stimulated Ca^{2+} entry both in MEG01 and HEK293 cells. Biotinylation analysis revealed that TPC1 and TPC2 are expressed in internal membranes. Finally, co-immunoprecipitation experiments indicated that endogenously expressed TPC2, but not TPC1, associates with STIM1 and Orai1, but not with TRPC1, in MEG01 cells with depleted intracellular Ca^{2+} stores, but not in resting cells. These results provide strong evidence for the modulation of SOCE by TPC2 involving *de novo* association between TPC2 and STIM1, as well as Orai1, in human cells.

1. Introduction

Store-operated calcium entry (SOCE) is a mechanism for Ca^{2+} influx controlled by the filling state of the intracellular Ca^{2+} stores [1]. The activation of SOCE has been reported to involved the participation of the stromal interaction molecule-1 (STIM1) [2–4], identified both as a membrane surface protein in stromal cells [5], and, predominantly, in the membranes of the endoplasmic reticulum (ER) [6] and certain acidic Ca^{2+} stores [7]. STIM1 located in the membrane of the internal Ca^{2+} stores expresses the Ca^{2+} binding domain (the EF hand motif) within the ER lumen. Hence, when the intraluminal Ca^{2+} concentration decreases STIM1 re-localizes at sites of close proximity to the plasma membrane (PM) and the ER membrane, allowing its association with members of the Orai and TRPC families [8–10].

SOCE has been reported to be modulated by the Ca^{2+} microdomains surrounding the elements of the SOCE machinery, both STIM1 and the store-operated channels (SOCs). Ca^{2+}-dependent inactivation of SOCs is influenced by mitochondria, which, either directly or through the activation of ATP-dependent Ca^{2+} clearance mechanisms [11], is able to buffer Ca^{2+} entering the cell [12] and STIM1 function is controlled by the intraluminal Ca^{2+} concentration, which is finely modulated by the interplay between closely located sarco/endoplasmic reticulum Ca^{2+} ATPases [13,14] and receptors for intracellular Ca^{2+}-mobilizing diffusible molecules.

In mammalian cells, intracellular Ca^{2+} mobilizing molecules include the inositol 1,4,5-trisphosphate (IP_3), cyclic ADP ribose or nicotinic acid adenine dinucleotide phosphate (NAADP). While a role for IP_3 and cyclic ADP ribose (ryanodine) receptors in the regulation of Ca^{2+} influx has long been proposed [15–17], a possible role for NAADP receptors in SOCE or other mechanisms for Ca^{2+} influx is unknown. Three NAADP-regulated receptors/channels have been identified as two-pore channels (TPC), with TPC1 and TPC2 both mediating NAADP-induced Ca^{2+} release, and TPC3, a pseudogene in humans and other primates [18,19]. TPC1 is predominantly localized in endosomes while TPC2 has been mostly identified in lysosome-related organelles [18,20], cellular compartments that have been presented as agonist-releasable Ca^{2+} stores in different cell types [7,21,22], with the ability to activate SOCE [23]. Despite, photoaffinity labeling of high affinity NAADP binding sites has demonstrated that this messenger molecule binds to an accessory protein rather than directly to TPCs both in mammalian cells [24] and sea urchin eggs [25], NAADP is the only known physiological modulator of TPCs.

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; BSA, bovine serum albumin; ER, endoplasmic reticulum; HBS, HEPES-buffered saline; TRPC1, canonical TRP1; IP_3, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PM, plasma membrane; PMSF, phenyl methyl sulphonyl fluoride; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SOCE, store-operated calcium entry; SOCs, store-operated channels; STIM1, stromal interaction molecule 1; TBST, tris-buffered saline with 0.1% Tween 20; TG, thapsigargin; TPC, two-pore channels

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In the present study we have investigated the possible role of TPC1 and TPC2 in SOCE in two unrelated mammalian cells, the megakaryoblastic cell line MEG01 and HEK-293. Our results indicate that, while TPC1 and TPC2 are expressed in both cell types, TPC1 knockdown was without effect in SOCE and silencing of TPC2 expression results in a significant attenuation of SOCE probably mediated by the interaction with STIM1 and Orai1, which suggest a functional role for TPC2 in the modulation of SOCE.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM) and calcein were from Invitrogen (Madrid, Spain). Thrombin, thapsigargin (TG), leupeptin, benzamidine, phenyl methyl sulphonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), rabbit anti-Orai1 antibody (C-terminal), rabbit anti-TPC2 antibody, nonidet P-40 and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Mouse anti-STIM1 antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, U.S.A.). Rabbit anti-TRPC1 polyclonal antibody was obtained from Antibodies-online GmbH (Aachen, Germany). Goat anti-TPC1 antibody, horseradish peroxidase-conjugated donkey anti-goat IgG antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) and hyperfilm ECL were from Amersham (Buckinghamshire, U.K.). Transpass COS/293 transfection reagent was from Izasa (Madrid, Spain). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U. K.). All other reagents were of analytical grade.

2.2. Cell culture

The human megakaryoblastic cell line MEG01 and HEK-293 cells were obtained from ATCC (Manassas, VA, USA) and cultured at 37 °C with a 5% CO2 in RPMI or DMEM media, respectively, supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μg/mL gentamycin.

shRNA, complementary oligonucleotides corresponding to a TPC1 target sequence (5′-CGAGCTGTATTTCATCATGAA-3′), TPC2 target sequence (5′-CAGGTGGGACCTCTGCATTGA-3′) or scramble RNA (scRNA) as control (5′-AATTCTCCGAACGTGTCACGT-3′) were kindly provided by Dr. Patel and described previously [20]. Cells were transiently transfected with shRNA using the Amaxa Nucleofection system (for MEG01 cells) or transpass COS/293 transfection reagent (for HEK-293 cells), and used 1–3 d after transfection as previously described [7].

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37 °C, centrifuged and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 mL aliquots using a Cary Eclipse spectrophotometer (Varian Ltd, Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique. 95% of the cells were viable in our preparations.

2.3. RT-PCR

Total RNA was extracted from MEG01 and HEK293 cells using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer specifications, and reverse-transcribed to single-strand cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Single-strand cDNA products were directly used for PCR amplification performed with an Eppendorff Mastercycler® thermal cycler (Eppendorf AG, Hamburg-Eppendorf, Germany). PCR reagents were purchased from TAKARA (Takara Bio Inc., Otsu, Shiga, Japan). PCR products were obtained using the following cycling conditions: 96 °C 2 min, followed by 35 cycles of 96 °C 15 s, 56 °C 25 s, 72 °C 30 s, and finished with 72 °C 10 min. The following primers were used to amplify human TPC (hTPC) cDNA transcripts; hTPC1_F: 5-ttctgtgttttgctttaggg-3 and hTPC1_R: 5-attccgcttccattagatcc-3: hTPC2_F: 5-gtttgacatggagagaa ccttgac-3 and hTPC2_R: 5-gatgaaaataactggcaatcagaacc-3 [20]. PCR products were separated by electrophoresis in 1.5% agarose gels (Roth, Germany) and the resulting bands were documented and subsequently isolated from gels using UltraClean® PCR Clean-Up Kit (MO BIO Laboratories Inc., Carlsbad, CA). The amplification of hTPC transcripts was confirmed by sequenciation of the PCR products (STAB- SAIUex, University of Extremadura, Spain).

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

Cells were loaded with fura-2 by incubation with 2 μM fura 2/AM for 45 min at 37 °C. Coverslips with cultured cells were mounted on a perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA) with image acquisition and analysis system for video microscopy (Hamamatsu Photonics, Hamamatsu, Japan). Cells were continuously superfused with HEPES-buffered saline containing (HBS in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45, and supplemented with 0.1% (w/v) BSA. Cells were alternatively excited with light from a xenon lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using Aquacosmos 2.5 software, (Hamamatsu Photonics, Hamamatsu, Japan).

Ca^{2+} entry was estimated using the integral of the rise in fura 2 fluorescence 340/380 nm ratio for 2 min after addition of $CaCl_2$. Ca^{2+} release was estimated using the integral of the rise in fura 2 fluorescence 340/380 nm ratio for 2 min after the addition of thrombin or TG [26].

To calculate the initial rate of Ca^{2+} elevation after the addition of Ca^{2+} to the medium, the traces were fitted to the equation $y = A + KX$, where K is the slope.

2.5. Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [27,28]. Briefly, 500 μL aliquots of cell suspensions (5×10^6 cells/mL) were lysed with an equal volume of Nonidet P-40 buffer, pH 8, containing 274 mM NaCl, 40 mM Tris, 20% glycerol, 4 mM EGTA, 2% nonidet P-40, 2 mM Na_3VO_4, 2 mM PMSF, 100 μg/mL leupeptin and 10 mM benzamidine. Aliquots of cell lysates (1 mL) were immunoprecipitated by incubation with 2 μg of anti-hTRPC1 antibody, anti-STIM1 antibody or anti-Orai1 antibody and 25 μL of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10% SDS-PAGE and separated proteins were electrophoretically transferred 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 TPC1 and TPC2 was achieved using the anti-TPC1 and anti-TPC2 antibodies diluted 1:500 in TBST overnight. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated for 45 min with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:10000 in TBST and then exposed to enhanced chemiluminiscence reagents for 5 min. Blots were then exposed to photographic films. The density of bands on the film was measured using scanning densitometry.

2.6. Biotinylation of membrane proteins

MEG01 cells and adherent monolayers of cultured HEK-293 cells grown in 75-cm^2 tissue culture dishes were washed three times

with HBS (without added serum or protein). 3 mL of Söerscen's buffer, pH7.0 (16 mM-Na_2HPO_4 and 114 mM-NaH_2PO_4) and containing 2.5 mg EZ-Link sulfo-NHS-LC-biotin was added, and the cells were incubated at 4 °C for 1 h. The biotinylation reaction was terminated by addition of Tris-base to a final concentration of 33 mM. Following biotinylation, cells were washed twice in Sörensen's buffer, disrupted using Nonidet P-40 buffer and sonicated. Cells were harvested by centrifugation (16000×*g*, 5 min at 4 °C). Samples were incubated with 25 μL streptavidin beads overnight at 4 °C and resuspended in Laemmli's buffer for subsequent analysis by Western blotting.

2.7. Statistical analysis

Analysis of statistical significance was performed using Student's *t*-test. $p<0.05$ was considered to be significant for a difference.

3. Results

3.1. TPC1 and TPC2 mRNA and protein are expressed in MEG01 and HEK293 cells

Two ubiquitously expressed TPC proteins have been described, TPC1 and TPC2. TPC3 has been presented as a pseudogene in humans and other primates [29,30] and appears to be absent in certain mammalian cells [19]. To identify expression of TPC1 and TPC2 in MEG01 and HEK293 cells we carried out RT-PCR. Semi-quantitative RT-PCR showed a single PCR band for TPC1 and TPC2 indicating significant expression of their mRNA transcripts in the human cell lines MEG01 and HEK293 (Fig. 1A). We have further investigated the protein expression of TPC1 and TPC2 in these cells. As shown in Fig. 1B and C, Western blotting of cell lysates with specific anti-TPC1 and TPC2 antibodies revealed the presence of both proteins in MEG01 and HEK293 cells.

3.2. Silencing TPC2 expression attenuates TG-induced Ca^{2+} influx

While the role of IP_3 and ryanodine receptors on Ca^{2+} influx has long been described [31–33], the involvement of TPCs in the regulation of Ca^{2+} fluxes across the PM remains unknown. We have investigated whether TPC1 and TPC2 channels modulate TG-evoked SOCE by silencing TPC1 and TPC2 expression. As shown in Fig. 2A, top panels, shTPC1 and shTPC2 reduced significantly the amount of these proteins detected in MEG01 cells by 81 and 77%, respectively, as compared with scRNA. Western blotting with anti-actin antibody revealed a similar amount of protein in all lanes (Fig. 2A, bottom panels).

Treatment of MEG01 cells with 1 μM TG resulted in a sustained increase in $[Ca^{2+}]_i$ due to passive Ca^{2+} efflux from the intracellular Ca^{2+} stores. Subsequent addition of Ca^{2+} to the extracellular medium induced a further increase in $[Ca^{2+}]_i$ indicative of SOCE. Our results indicate that shTPC1 and shTPC2 did not significantly modify TG-evoked Ca^{2+} release from the intracellular stores, as compared to cells treated with scRNA (Fig. 2B and C; $n=6$); thus suggesting that TPC1 and TPC2 silencing does not alter the ability of MEG01 cells to accumulate Ca^{2+} in internal organelles. TPC1 silencing had a negligible effect, if any, in TG-induced SOCE in these cells. As shown in Fig. 2B, the integral of the rise in $[Ca^{2+}]_i$ for 2 min after the addition of Ca^{2+} to the medium in shTPC1-treated cells was 97% of control. Furthermore, TPC1 silencing did not modify the initial rate of increase in $[Ca^{2+}]_i$ upon Ca^{2+} addition (the initial slope of $[Ca^{2+}]_i$ elevation was 0.0482 ± 0.0067 in control cells and 0.0439 ± 0.0049 in shTPC1-treated cells ($n=6$)). Interestingly, silencing of TPC2 expression significantly attenuated both the extent of TG-induced SOCE (the integral of the rise in $[Ca^{2+}]_i$ for 2 min after the addition of Ca^{2+} to the medium in shTPC2-treated cells was 67% of control; Fig. 2C; $p<0.05$; $n=6$), as well as the initial rate of increase in $[Ca^{2+}]_i$ upon Ca^{2+} addition (the initial slope was 0.0301 ± 0.0037 in shTPC2-treated cells; Fig. 2C; $p<0.05$; $n=6$).

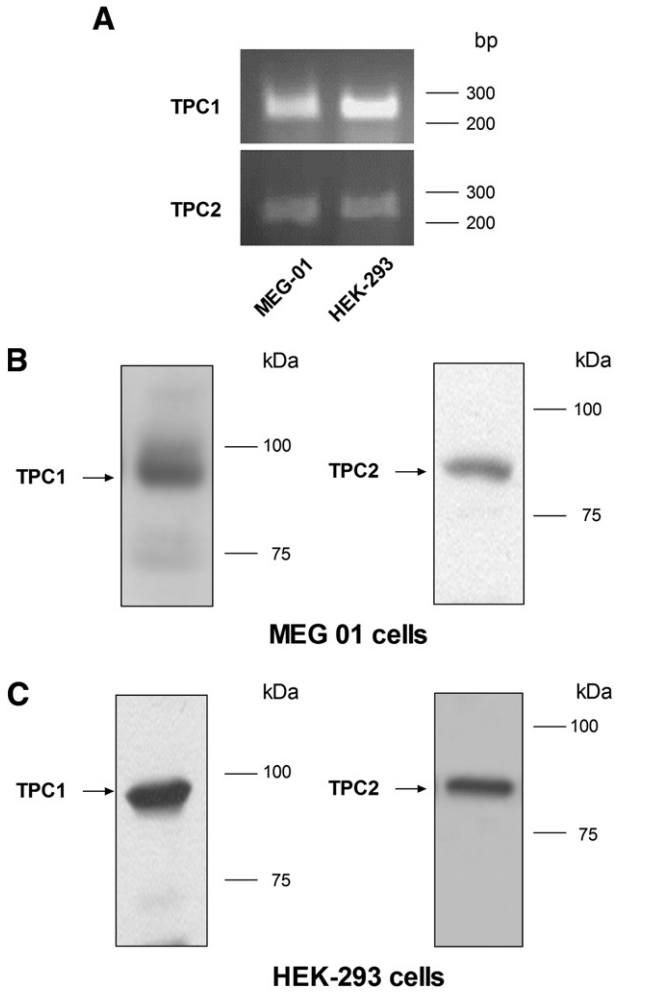

Fig. 1. TPC1 and TPC2 are expressed in MEG01 and HEK-293 cells. Non-stimulated MEG01 and HEK-293 cells were lysed and mRNA expression was determined by RT-PCR. bp: pairs of bases. (A). Alternatively, lysates were subjected to Western blotting using specific anti-TPC1 and anti-TPC2 antibodies (B and C). The results represented three independent experiments.

Similar results were found when we investigated the role of TPCs in agonist-evoked Ca^{2+} mobilization in MEG01 cells. As shown in Fig. 3, treatment of cells with thrombin (1 U/mL) resulted in a transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from the intracellular stores mediated by the generation of diffusible messengers. Subsequent addition of Ca^{2+} leads to a further $[Ca^{2+}]_i$ elevation attributed to receptor-operated Ca^{2+} entry mechanisms, including SOCE. Thrombin-evoked Ca^{2+} entry was transient in contrast to the sustained effect induced by TG, which does not allow intracellular stores to refill. As shown in Fig. 3, MEG01 cells treatment with shTPC1 and shTPC2 did not significantly modify thrombin-stimulated Ca^{2+} release from the intracellular compartments, as compared to cells treated with scRNA. As for TG-evoked SOCE, silencing of TPC2 expression resulted in attenuated initial rate of $[Ca^{2+}]_i$ elevation and reduced extent of Ca^{2+} entry. The initial slope of $[Ca^{2+}]_i$ elevation after stimulation with thrombin was 0.0578 ± 0.0046 in control cells and 0.0431 ± 0.0042 in shTPC2-treated cells; Fig. 3C; $p<0.05$; $n=6$. In contrast, and in agreement with the results reported for TG, TPC1 silencing was without effect on thrombin-evoked Ca^{2+} entry (the initial slope of $[Ca^{2+}]_i$ elevation after stimulation with thrombin was 0.0591 ± 0.0039 in shTPC1-treated cells; Fig. 3B; $n=6$).

To confirm the findings observed in MEG01 cells we investigated the effect of TPC1 and TPC2 silencing in TG-induced SOCE in HEK293 cells. Treatment of HEK293 cells with 1 μM TG resulted in a sustained increase in $[Ca^{2+}]_i$ due to Ca^{2+} leak from the intracellular

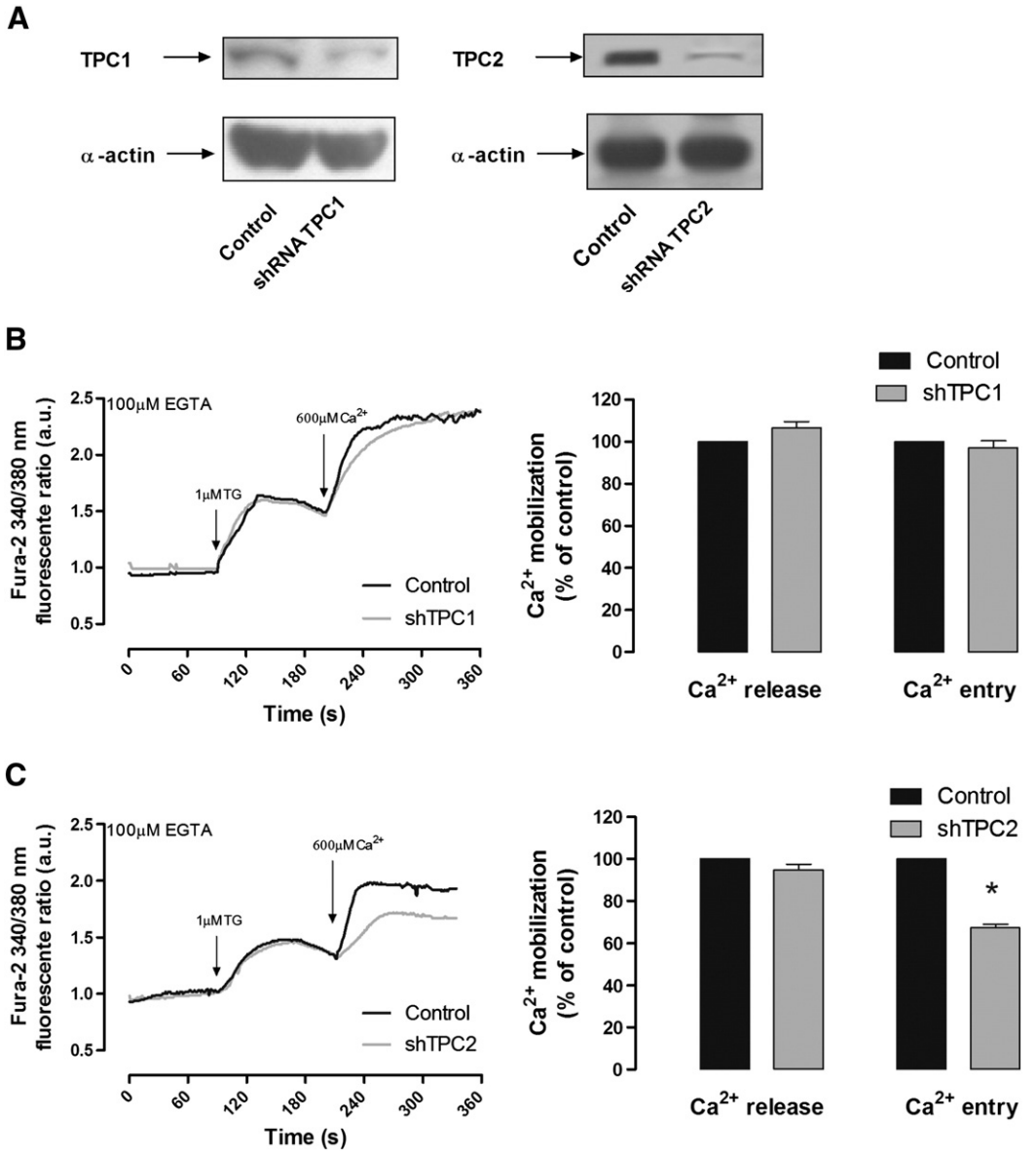

Fig. 2. Silencing TPC2 expression attenuates the rate and extent of Ca^{2+} influx during SOCE in MEG01 cells. MEG01 cells were transfected with shTPC1, shTPC2 or control vector, as indicated, and used after 48 h for protein detection. A, Western blotting showing the expression levels of TPC1 and TPC2, which was significantly reduced in lysates from cells transfected with shTPC1 (left panel) or shTPC2 (right panel). Bottom panel, Western blotting with anti-α actin antibody for protein loading control. B and C, MEG01 cells were transfected with shTPC1 (B), shTPC2 (C) or control vector, as indicated. Cells were loaded with fura-2 and stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 μM TG, followed by addition of $CaCl_2$ (final concentration 600 μM) to initiate Ca^{2+} entry. Traces are representative of 6 independent experiments. Histograms represent TG-induced Ca^{2+} release and Ca^{2+} entry expressed as percentage of Control (cells not treated with shTPC1 or shTPC2). Ca^{2+} release and entry was determined as described in Materials and methods. Values are means ± S.E.M.; significance values indicate differences compared with TG-treated cells in the absence of shTPC2. *$p<0.05$.

Ca^{2+} compartments. Subsequent addition of Ca^{2+} to the external medium resulted in a further increase in $[Ca^{2+}]_i$ indicative of SOCE. As for MEG01 cells, our results indicate that treatment of HEK293 cells with shTPC1 and shTPC2 did not significantly alter TG-evoked Ca^{2+} release from the intracellular stores, as compared to cells treated with scRNA (Fig. 4A and B; $n=6$); thus indicating that TPC1 and TPC2 silencing does not interfere with the ability of HEK293 cells to accumulate Ca^{2+} into TG-sensitive stores. In agreement with results reported above, TPC1 silencing did not significantly modify either the extent of TG-induced SOCE (the integral of the rise in $[Ca^{2+}]_i$ for 2 min after the addition of Ca^{2+} to the medium in shTPC1-treated cells was 111% of control) or the initial rate of increase in $[Ca^{2+}]_i$ upon Ca^{2+} addition (the initial slope of $[Ca^{2+}]_i$ elevation was 0.0401 ± 0.0033 in control cells and 0.0432 ± 0.0042 in shTPC1-treated cells; Fig. 4A; $n=6$). In contrast, silencing of TPC2 expression significantly attenuated both the extent of TG-induced SOCE (the integral of the rise in $[Ca^{2+}]_i$ for 2 min after the addition of Ca^{2+} to the medium in shTPC2-treated cells was 55% of control; Fig. 4B; $p<0.05$; $n=6$), as well as the initial rate of increase in $[Ca^{2+}]_i$ upon Ca^{2+} addition (the initial slope was 0.0211 ± 0.0027 in shTPC2-treated cells; Fig. 4B; $p<0.05$; $n=6$).

3.3. TPC1 and TPC2 are not expressed in the plasma membrane

To investigate whether the modulatory effect of TPC2 on SOCE and agonist-stimulated Ca^{2+} entry is mediated by their presence in the PM, we assessed the surface expression of TPC1 and TPC2 in resting MEG01 and HEK293 cells by biotinylation of PM proteins and collection with streptavidin-coated agarose beads. SDS-PAGE and Western blotting were used to identify TPC1, TPC2 and Orai1, a SOC subunit

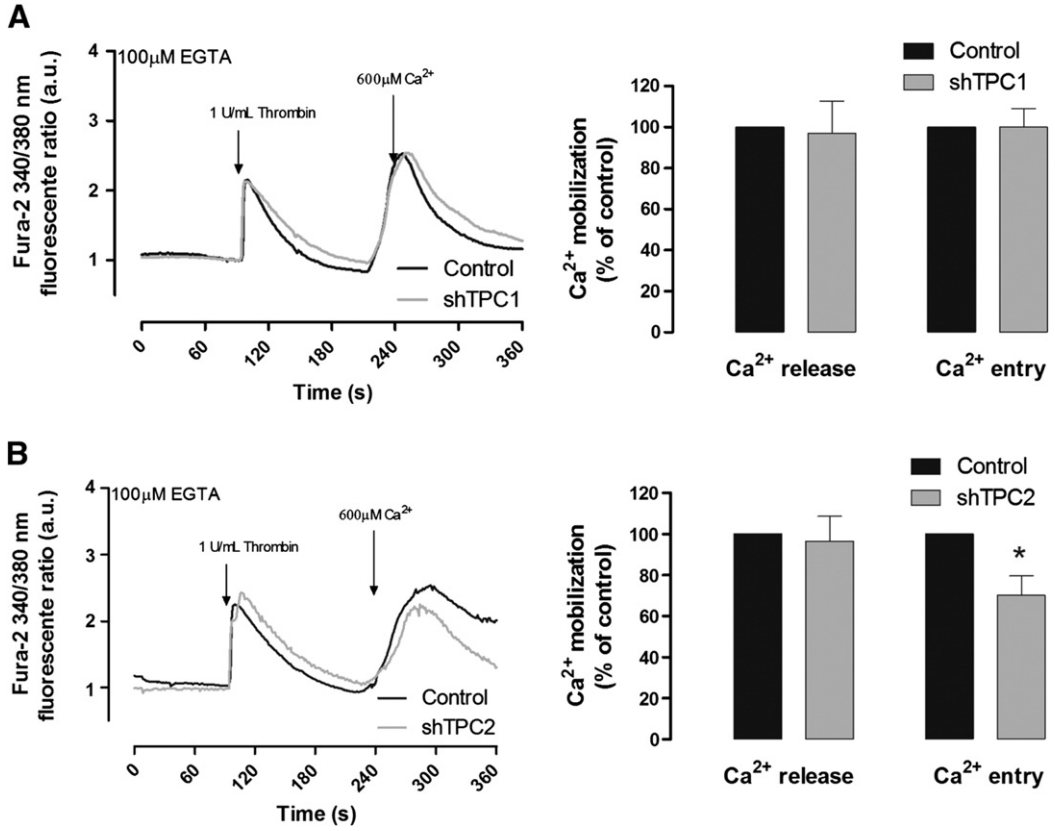

Fig. 3. Silencing TPC2 expression attenuates the rate and extent of Ca^{2+} influx stimulated by thrombin in MEG01 cells. MEG01 cells were transfected with shTPC1, shTPC2 or control vector, as indicated, and used after 48 h for protein detection. A and B, MEG01 cells were transfected with shTPC1 (A), shTPC2 (B) or control vector, as indicated. Cells were loaded with fura-2 and stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 U/mL thrombin, followed by addition of $CaCl_2$ (final concentration 600 μM) to initiate Ca^{2+} entry. Traces are representative of 6 independent experiments. Histograms represent thrombin-induced Ca^{2+} release and Ca^{2+} entry expressed as percentage of Control (cells not treated with shTPC1 or shTPC2). Ca^{2+} release and entry was determined as described in Materials and methods. Values are means ± S.E.M.; significance values indicate differences compared with TG-treated cells in the absence of shTPC2. $^*p<0.05$.

located in the PM [34,35] determined as positive control. As shown in Fig. 5, analysis of biotinylated proteins shows that Orai1 is present in the PM in resting MEG01 and HEK-293 cells. In contrast, we were unable to detect TPC1 and TPC2 in the PM under the same experimental conditions (Fig. 5; $n=4$); thus indicating that TPC1 and TPC2 are expressed in the membrane of internal organelles, as previously described [18,36,37].

3.4. TPC2 co-immunoprecipitates with STIM1 and Orai1 but not with TRPC1 in MEG01 cells

In order to explore the mechanism underlying the modulation of SOCE by TPC2 we investigated its association with STIM1, Orai1 and TRPC1 by looking for co-immunoprecipitation from MEG01 lysates. Immunoprecipitation and subsequent SDS/PAGE and Western blotting were conducted using resting cells and cells in which the intracellular Ca^{2+} stores had been depleted by 3 min of pretreatment with TG (1 μM) in the absence of extracellular Ca^{2+} (100 μM EGTA). After immunoprecipitation with the anti-STIM1 antibody, Western blotting revealed the presence of TPC2 in samples from store-depleted, but not resting cells (Fig. 6B, top panel; $n=5$), while TPC1 was undetectable in samples from either resting or store-depleted cells (Fig. 6A, top panel; $n=5$), which is consistent with a role for TPC2, but not TPC1, in the regulation of SOCE, as reported above. Western blotting with anti-STIM1 antibody confirmed a similar content of this protein in all lanes (Fig. 6A and B, bottom panels). We also conducted experiments on immunoprecipitating MEG01 cell lysates under resting or TG-stimulated conditions with either anti-Orai1 or anti-TRPC1 antibody. After immunoprecipitation with anti-Orai1, TPC2 was detected in samples from store-depleted, but not resting MEG01 cells (Fig. 6C, top panel; $n=5$); however, after immunoprecipitation with anti-TRPC1 TPC2 was undetectable in samples from control or TG-treated samples (Fig. 6D, top panel; $n=5$). Immunoblot analysis of anti-Orai1 or anti-TRPC1 immunoprecipitates with the same antibodies, as appropriate, revealed a similar content of these proteins in the relevant lanes (Fig. 6C and D, bottom panels; $n=5$).

4. Discussion

Cytosolic Ca^{2+} concentration is modified by a vast number of agonists in order to regulate cellular function. Agonist-receptor interaction leads to the generation of distinct intracellular Ca^{2+}-releasing molecules, among them, NAADP has been identified as the most potent Ca^{2+}-mobilizing messenger described [38,39]. NAADP has been reported to regulate different ion channels, including the type I ryanodine receptor [40] and TRP-ML1 (transient receptor potential channel, subtype mucolipin 1) [41]. More recently, a number of studies demonstrated that TPCs are NAADP-regulated Ca^{2+} channels [18,20,42]. TPCs are located in endosomes and lysosome-related organelles, although TPC2 has been reported as the major lysosomal targeted isoform [43]. SOCE is a receptor-operated Ca^{2+} influx mechanism controlled by the amount of Ca^{2+} accumulated into agonist-sensitive compartments, mostly described as the ER and lysosomal-related (acidic) organelles [23,44], both expressing the Ca^{2+} sensor STIM1 [3,7]. However, despite SOCE is activated by discharge of the lysosomal-related organelles, where Ca^{2+} efflux via the TPCs/NAADP

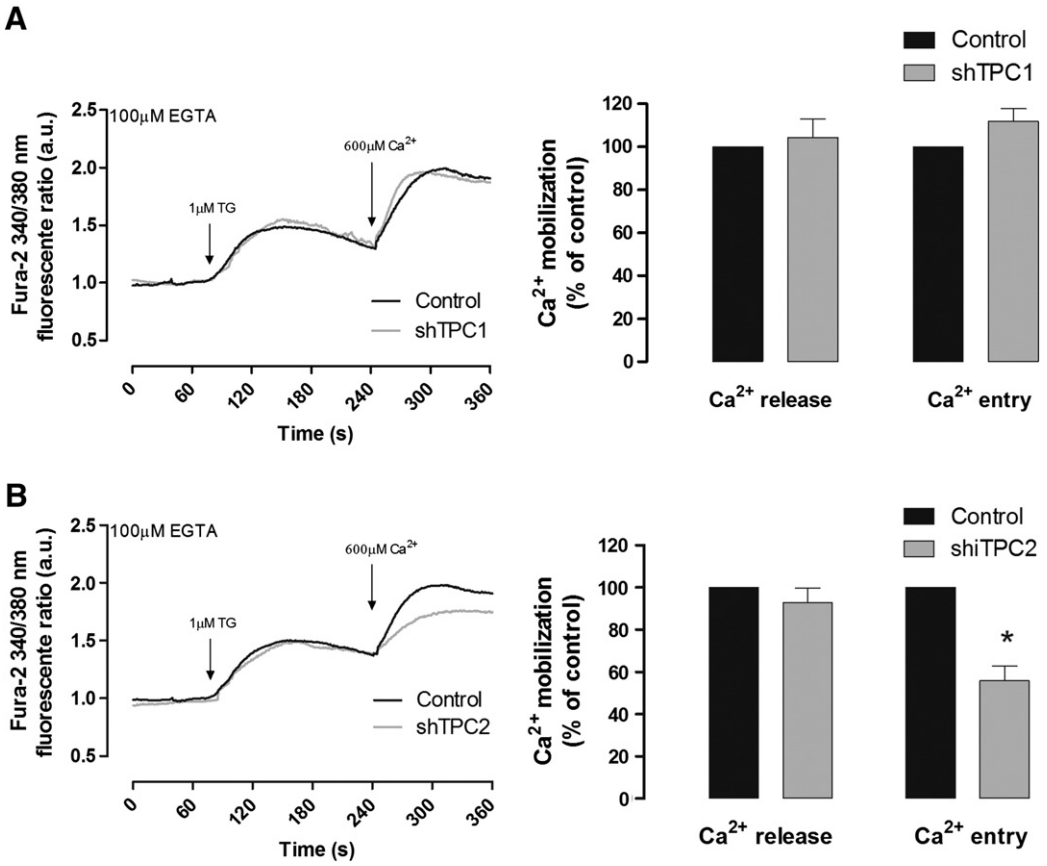

Fig. 4. Silencing TPC2 expression attenuates the rate and extent of Ca^{2+} influx during SOCE in HEK293 cells. HEK293 cells were transfected with shTPC1, shTPC2 or control vector, as indicated, and used after 48 h for protein detection. A and B, cells were transfected with shTPC1 (A), shTPC2 (B) or control vector, as indicated. Cells were loaded with fura-2 and stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 μM TG, followed by addition of $CaCl_2$ (final concentration 600 μM) to initiate Ca^{2+} entry. Traces are representative of 6 independent experiments. Histograms represent TG-induced Ca^{2+} release and Ca^{2+} entry expressed as percentage of Control (cells not treated with shTPC1 or shTPC2). Ca^{2+} release and entry was determined as described in Materials and methods. Values are means ± S.E.M.; significance values indicate differences compared with TG-treated cells in the absence of shTPC2. $^{*}p<0.05$.

pathway plays a major role, a possible modulatory effect of TPCs on SOCE has not been evaluated yet. Our results show, for the first time, that silencing of the expression of endogenous TPC2 is able to attenuate both the initial rate of Ca^{2+} entry and the extent of SOCE stimulated by TG, without affecting the ability of cells to accumulate Ca^{2+} into the intracellular stores, in two unrelated human cell lines, the megakaryoblastic MEG01 cells and HEK293 cells. In contrast, significant attenuation of endogenous TPC1 expression in both cell lines was unable to modify either TG-induced Ca^{2+} release from the stores or TG-stimulated SOCE. TG was used to induce passive store depletion by inhibition of Ca^{2+} reuptake into the agonist-sensitive stores by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), whose expression has been demonstrated in the ER, endosomes and lysosomal-related organelles [45]. We have further found that silencing TPC2, but not

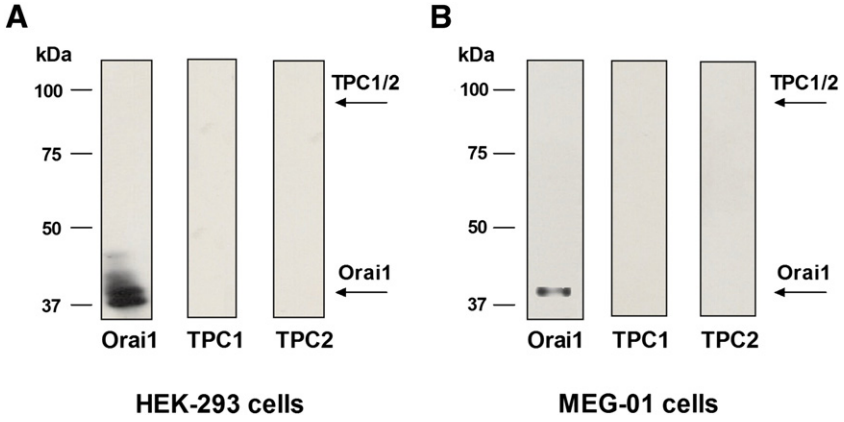

Fig. 5. TPC1 and TPC2 are not expressed in the plasma membrane in MEG01 and HEK293 cells. HEK293 (A) and MEG01 cells (B) under resting conditions were incubated with ice-cold Söerscen's buffer and cell surface proteins were labeled by biotinylation, extracted with streptavidin-coated agarose beads, and analyzed by SDS-PAGE and Western blotting using the anti-Orai1, anti-TPC1 and anti-TPC2 antibodies. Positions of molecular mass markers are shown on the left.

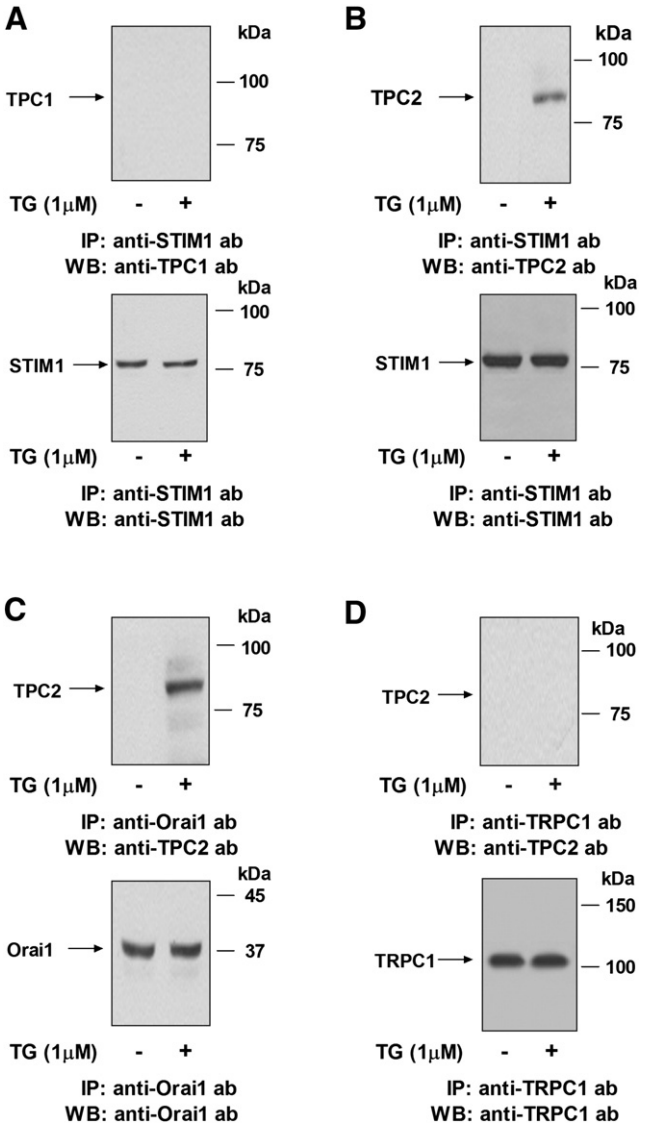

Fig. 6. TPC2 co-immunoprecipitates with STIM1 and Orai1 in MEG01 cells. Cells were stimulated with 1 μM TG for 3 min or the vehicle, as control, and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody (A and B), anti-Orai1 antibody (C) or anti-TRPC1 antibody (D). Immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-TPC1 antibody (A) or anti-TPC2 antibody (B–D). Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panel shows results from one experiment representative of 4 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel.

TPC1, expression reduces Ca^{2+} entry by the physiological agonist thrombin. The lack of effect of alternative silencing of TPC1 and TPC2 expression on thrombin-stimulated Ca^{2+} release might be attributed to different factors: i) thrombin is unable to generate significant amounts of NAADP to induce a detectable signal; ii) the effect of the attenuation of TPC1 or TPC2 expression on thrombin-evoked Ca^{2+} release is overcome by the actions of other intracellular channels, including the IP_3 or ryanodine receptors (MEG01 cells have been reported to express IP_3 receptors types I, II and III [46] and the type III ryanodine receptor [47]).

We have explored the mechanism underlying the modulation of SOCE by TPC2 and tested the presence of TPCs in the PM, where the SOCs are located. Biotinylation analysis confirmed that TPCs are not expressed in the PM. Our results also report for the first time that discharge of the intracellular Ca^{2+} stores using TG leads to the association of TPC2 with the Ca^{2+} sensor STIM1 and Ca^{2+} channel Orai1; however, we did not detect association of TPC2 with TRPC1. The interaction of TPC2 with STIM1 and Orai1 was only found in cells where the Ca^{2+} stores had been depleted using TG and not in resting, undepleted, cells, which suggest that this is a *de novo* association whose activation shows parallelism with that of SOCE. The association of TPC2 with STIM1 upon store depletion strongly suggests that TPC2 plays a relevant role in the regulation of SOCE. Although speculative, rapid Ca^{2+} efflux mediated by TPC2 in the close proximity of STIM1 might influence the rate and extent of SOCE activation by the latter, which would explain the attenuation of the initial rate and extent of SOCE found upon silencing TPC2 expression. The TPC2-Orai1 association might occur either directly or indirectly and mediated by STIM1, and this interaction might also be important for the regulation of SOCE by TPC2.

The involvement of TPC2, but not TPC1, in the modulation of SOCE might be attributed to the distinct distribution of TPCs in human cells, with TPC2 predominantly located in lysosomal-related organelles [18,20,43]. In addition, relevant functional differences between TPC1 and TPC2 have been described. While Ca^{2+} release through both receptor/channels are regulated by NAADP, the subsequent amplification of this Ca^{2+} signal by IP_3 receptors, which are also involved in the regulation of SOCE [48], is more tightly coupled for TPC2 [29].

Our findings provide the first evidence for a role for TPC2 in the modulation of SOCE. The new role of TPC2 might be mediated by colocalization with STIM1 in internal stores, which might modify the kinetics of STIM1-Ca^{2+} binding and, thus, Ca^{2+} sensing and the activation rate and extent of SOCE.

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