

TRPC3 Regulates Agonist-stimulated Ca^{2+} Mobilization by Mediating the Interaction between Type I Inositol 1,4,5-Trisphosphate Receptor, RACK1, and Orai1*

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Geoffrey E. Woodard[†], José J. López^{§1}, Isaac Jardín^{§2}, Ginés M. Salido[§], and Juan A. Rosado^{§3}

From the [†]NIDDK, National Institutes of Health, Bethesda, Maryland 20892-2560 and the [§]Department of Physiology, Cell Physiology Research Group, University of Extremadura, 10071 Cáceres, Spain

There is a body of evidence suggesting that Ca^{2+} handling proteins assemble into signaling complexes required for a fine regulation of Ca^{2+} signals, events that regulate a variety of critical cellular processes. Canonical transient receptor potential (TRPC) and Orai proteins have both been proposed to form Ca^{2+} -permeable channels mediating Ca^{2+} entry upon agonist stimulation. A number of studies have demonstrated that inositol 1,4,5-trisphosphate receptors (IP_3Rs) interact with plasma membrane TRPC channels; however, at present there is no evidence supporting the interaction between Orai proteins and IP_3Rs . Here we report that treatment with thapsigargin or cellular agonists results in association of Orai1 with types I and II IP_3Rs . In addition, we have found that TRPC3, RACK1 (receptor for activated protein kinase C-1), and STIM1 (stromal interaction molecule 1) interact with Orai1 upon stimulation with agonists. TRPC3 expression silencing prevented both the interaction of Orai1 with TRPC3 and, more interestingly, the association of Orai1 with the type I IP_3R , but not with the type II IP_3R , thus suggesting that TRPC3 selectively mediates interaction between Orai1 and type I IP_3R . In addition, TRPC3 expression silencing attenuated ATP- and CCh-stimulated interaction between RACK1 and the type I IP_3R , as well as Ca^{2+} release and entry. In conclusion, our results indicate that agonist stimulation results in the formation of an Orai1-STIM1-TRPC3-RACK1-type I IP_3R complex, where TRPC3 plays a central role. This Ca^{2+} signaling complex might be important for both agonist-induced Ca^{2+} release and entry.

Cellular stimulation by agonists results in a rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$),⁴ an event that is essential for a large number of cellular processes. Agonist-evoked Ca^{2+} mobilization consists of two components: Ca^{2+} release from finite

intracellular stores and Ca^{2+} entry. Often the increase in $[\text{Ca}^{2+}]_i$ resulting from Ca^{2+} entry is of major magnitude than Ca^{2+} release, and is required for full activation of cellular functions (1, 2).

Agonist receptor activation results in the hydrolysis of membrane phosphoinositides by phospholipase C and the generation of Ca^{2+} mobilizing messenger inositol 1,4,5-trisphosphate (IP_3), which, upon activation of different IP_3 receptors (IP_3Rs), releases Ca^{2+} from non-mitochondrial intracellular Ca^{2+} stores (3). In non-excitabile cells, receptor occupation results in activation of two separate pathways for Ca^{2+} entry, named receptor-operated Ca^{2+} entry (ROCE) and capacitative or store-operated Ca^{2+} entry (SOCE). The latter is a major mechanism for Ca^{2+} influx regulated by the filling state of the intracellular Ca^{2+} stores (4), a mechanism where the stromal interaction molecule (STIM) 1 has been demonstrated to act as the transmembrane endoplasmic reticulum Ca^{2+} sensor (5–8). The nature of the plasma membrane Ca^{2+} permeable channels involved both in ROCE and SOCE are still under investigation but most studies have presented Orai1 as a putative SOC channel (9–14) and transient receptor potential (TRP) proteins as candidates to mediate both SOCE and ROCE (15–20). These channels have been shown to take part in signaling complexes, including the protein STIM1, which might be essential for the activation mode of the channel (19, 21–23).

In addition, a functional interaction between IP_3Rs and human TRP channels has been demonstrated by different approaches in several cell types, including human platelets endogenously expressing TRPC1 and IP_3Rs (24, 25), human embryonic kidney (HEK)-293 cells stably expressing hTRP3 (26) or TRPC1–6 proteins (27), and HEK293T transiently expressing different TRP proteins (28). IP_3Rs have also been shown to be required for activation of TRPC1 in vascular smooth muscle cells (29) and for the IP_3 -dependent miniature Ca^{2+} channels (I_{min}), a Ca^{2+} -selective channel activated by store depletion, in excised membrane patches from A431 human carcinoma cells (30).

A recent study has reported that TRPC3 regulates IP_3R function by mediating interaction between IP_3R and the scaffolding protein RACK1 (receptor for activated protein kinase C-1), a protein that plays a key role in transduction of plasma membrane signals to downstream effectors (31, 32), thus regulating agonist-induced Ca^{2+} release (33). Hence, in the present study we have investigated whether this complex is also important for agonist-induced Ca^{2+} entry with the participation of proteins involved in Ca^{2+} entry such as Orai1 and STIM1. We describe for the first time association

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² Supported by DGI fellowship BFU2007-60104.

³ To whom correspondence should be addressed. Tel.: 34-927257139; Fax: 34-927257110; E-mail: jarosado@unex.es.

⁴ The abbreviations used are: $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; IP_3 , inositol 1,4,5-trisphosphate; IP_3R , inositol 1,4,5-trisphosphate receptor; ROCE, receptor-operated calcium entry; SOCE, store-operated calcium entry; STIM1, stromal interaction molecule 1; TG, thapsigargin; TRP, transient receptor potential; DAPI, 4',6-diamidino-2-phenylindole; CCh, carbachol; HEK, human embryonic kidney; shRNA, short hairpin RNA; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

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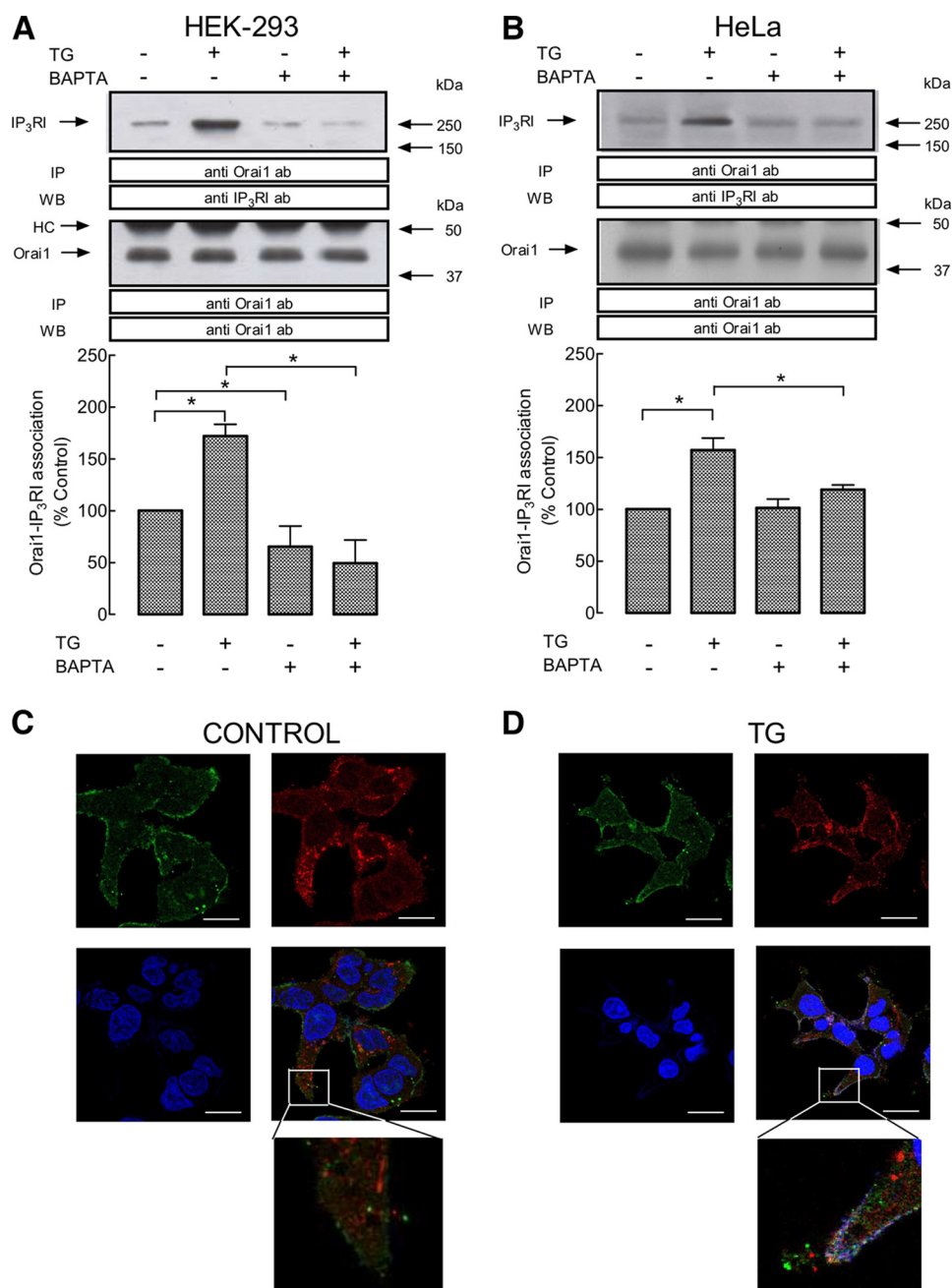


FIGURE 1. Interaction of Orai1 with type I IP₃R in HEK293 and HeLa cells. *A* and *B*, HEK (*A*) and HeLa cells (*B*) were either loaded with dimethyl-BAPTA or left treated, as indicated, and then suspended in a medium containing 1 mM Ca²⁺ or a Ca²⁺-free medium (BAPTA-loaded cells; 100 μM EGTA was added). Cells were then stimulated with TG (1 μM) for 3 min or left untreated and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-Orai1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-IP₃RI antibody. Membranes were reprobbed with the anti-Orai1 antibody for protein loading control. The panel shows results from one experiment representative of five. Molecular masses indicated on the right were determined using molecular mass markers run in the same gel. Histograms represent the quantification of Orai1-IP₃RI association under different experimental conditions. Results are expressed as mean ± S.E. and presented as percentage of control (non-stimulated cells not loaded with dimethyl-BAPTA). *, *p* < 0.05. HC, heavy chain of the immunoglobulin used for immunoprecipitation. *C* and *D*, confocal images of resting (*C*) and TG-treated (*D*) HEK293 cells immunostained with anti-Orai1 and anti-IP₃RI antibodies followed by Alexa Fluor 488- and 568-conjugated secondary antibodies, respectively, or DAPI. An overlay of the three images is depicted on the bottom right-hand image. Cells were stimulated in the absence (control) or presence of 1 μM TG, as indicated, in a medium containing 1 mM Ca²⁺. Scale bar, 10 μm. Insets depict a zoom of two representative areas. Error bars, S.E.

of the Ca²⁺ permeable channel Orai1 with the type I and II IP₃ receptors. The former appears to be mediated by TRPC3 proteins, which also mediates their interaction with RACK1.

tion and resuspended in HEPES-buffered saline.

Cell transfection with shTRPC3, shTRPC1, and shOrai1 was performed as described previously (33, 34) using

This protein complex might play a functional role in agonist-induced Ca²⁺ mobilization.

EXPERIMENTAL PROCEDURES

Materials—ATP, thapsigargin (TG), leupeptin, benzamide, ionomycin, phenylmethylsulfonyl fluoride, dimethyl-BAPTA-AM, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), carbachol (CCh), anti-G actin antibody, bovine serum albumin, and anti-Orai1 antibody (C-terminal) were from Sigma. Anti-TRPC3 (N-terminal) and anti-TRPC1 (C-terminal) antibodies were from Abcam (Cambridge, UK). Anti-type I IP₃ receptor and anti-type II IP₃ receptor antibodies, anti-RACK1 antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG, and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-type III IP₃ receptor antibody and anti-STIM1 antibody were from BD Biosciences. Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) and hyperfilm ECL were from Amersham Biosciences. Fura 2-AM, Alexa Fluor 488- and 568-conjugated secondary antibodies, and DAPI were from Invitrogen. Enhanced chemiluminescence detection reagents were from Pierce. All other reagents were of analytical grade.

Cell Culture and Transfection—Human HeLa and HEK293 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium, and supplemented with 10% heat-inactivated fetal bovine serum in a 37 °C incubator with 5% CO₂. The experiments were performed in HEPES-buffered saline containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO₄, pH 7.45. For dimethyl-BAPTA loading, cells were incubated for 30 min at 37 °C with 10 μM dimethyl-BAPTA-AM. Cells were then collected by centrifuga-

shTRPC3, shTRPC1, and shOrai1 kindly provided by Dr. Ambudkar. For shTRPC3, the sense sequence was 5'-CACCGTGATGTGGTCTGAATGTAACGAATTACATTCAGACCACATCAC-3', and the antisense sequence was 5'-AAAAGTGATGTGGTCTGAATGTAATTCGTTACATT-CAGACCACATCAC-3'. For the shRNA targeting human TRPC1, the sense sequence was 5'-CACCGGGTGACTTTATATGGTTTCGAAAACCATATAATAGTCACCC-3', and the antisense sequence was 5'-AAAAGGGTGACTATTATATGGTTTTCGAAACCATATAATAGTCACCC-3'. For the shOrai1, the sense sequence was 5'-CACCTCACTGGT-TAGCCATAAGACGAATCTTATGGCTAACCAGTGA-3', and the antisense sequence was 5'-AAAACCTTTACACGCTAGATGGTtctgTCTTATGGCTAACCAGTGA-3'. These sequences were synthesized and hybridized as previously described (33, 34). Plasmids were used for silencing experiments at 1 $\mu\text{g}/\mu\text{l}$.

Immunoprecipitation—Cell suspension aliquots (500 μl) were treated as described and lysed with an equal volume of lysis buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% Triton X-100, 2 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, 100 $\mu\text{g}/\text{ml}$ of leupeptin, and 10 mM benzamide. Protein immunoprecipitation was achieved by incubating samples with 2 μg of the specific antibody and protein A-agarose overnight at 4 °C.

Western Blotting—Western blotting was performed as described previously (35). Briefly, proteins were separated by 10% SDS-PAGE and electrophoretically transferred for 2 h at 0.8 mA/cm², in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffordshire, UK) onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of Orai1, TRPC3, RACK1, G actin, or types I, II, or III IP₃ receptors was achieved using the anti-Orai1, anti-TRPC3, anti-RACK1, anti-STIM1, anti-G actin, anti-type I IP₃ receptor, anti-type II IP₃ receptor, or anti-type III IP₃ receptor antibodies diluted 1:1000 in TBST for 2 h or the anti-TRPC1 antibody diluted 1:200 in TBST for 2 h. To detect the primary antibody, blots were incubated for 1 h with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:10,000 in TBST and then exposed to enhanced chemiluminescence reagents for 4 min. Blots were then exposed to photographic films. The density of bands on the film was measured using a scanning densitometry.

Immunofluorescence—Cultured cells were fixed using 3% paraformaldehyde (in phosphate-buffered saline) for 10 min at room temperature. The cells were then permeabilized in phosphate-buffered saline containing 0.025% (v/v) Nonidet P-40 detergent for 10 min at 4 °C. Samples were incubated with rabbit anti-Orai1 or goat anti-type I IP₃ receptor antibodies overnight at room temperature in phosphate-buffered saline containing 0.5% bovine serum albumin as blocking agent, followed by incubation with Alexa Fluor 488- and 568-conjugated secondary antibodies and DAPI for 1 h. The samples were examined using a Zeiss LSM 510 confocal microscope. For quantitative colocalization analysis the overlap coefficient according to Manders *et al.* (36) was calculated using Image J software.

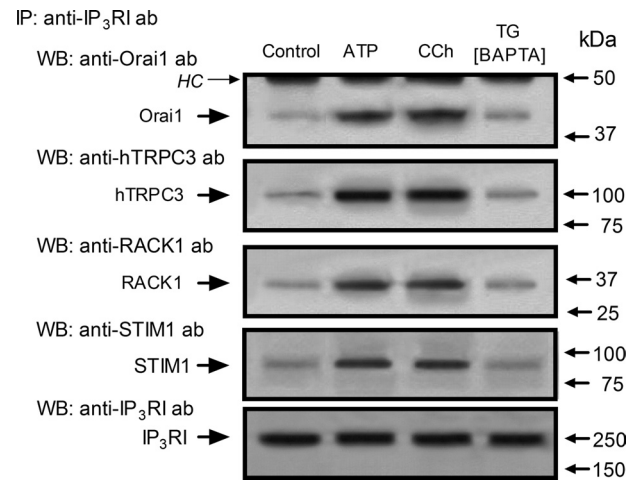


FIGURE 2. Interaction of type I IP₃R with Orai1, STIM1, TRPC3, and RACK1 in HEK293. HEK293 cells were suspended in a medium containing 1 mM Ca²⁺ and then stimulated with ATP (10 μM) or CCh (100 μM) for 3 min and lysed. Alternatively, cells were loaded with dimethyl-BAPTA, suspended in Ca²⁺-free medium (100 μM EGTA was added), and then stimulated with TG (1 μM) for 3 min and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-type I IP₃R antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting (WB) with specific anti-Orai1, anti-TRPC3, anti-RACK1, or anti-STIM1 antibodies. Membranes were re-probed with the anti-type I IP₃R antibody for protein loading control. The panels show results from one experiment representative of five. Molecular masses indicated on the right were determined using molecular mass markers run in the same gel. HC, heavy chain of the immunoglobulin used for immunoprecipitation.

Measurement of Intracellular Free Calcium Concentration ([Ca²⁺]_i)—Cells were loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37 °C. Fluorescence was recorded from 2-ml aliquots of magnetically stirred cellular suspension (10⁶ cells/ml) at 37 °C using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in [Ca²⁺]_i were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to an established method (37).

Ca²⁺ release by ATP or CCh was estimated using the integral of the rise in [Ca²⁺]_i for 3 min after the addition of the agonist (38). Ca²⁺ entry was estimated using the integral of the rise in [Ca²⁺]_i for 2.5 min after addition of CaCl₂ (39). Ca²⁺ entry was corrected by subtraction of the [Ca²⁺]_i elevation due to leakage of the indicator.

Statistical Analysis—Analysis of statistical significance was performed using Student's *t* test. The difference was considered statistically significant when at least *p* < 0.05.

RESULTS

Thapsigargin Enhances the Association of Orai1 with Type I IP₃ Receptor—We have investigated the possible association between the plasma membrane protein Orai1 and the type I IP₃R in two unrelated human cell lines, HEK293 and HeLa cells, by looking for co-immunoprecipitation from cell lysates. Immunoprecipitation and subsequent SDS-PAGE and Western blotting were conducted using resting cells and cells treated with TG, a specific inhibitor of SERCA (40) that promotes passive Ca²⁺ efflux from the endoplasmic reticulum and, subsequently, rises in [Ca²⁺]_i. As depicted in Fig. 1, *A* and *B*, upper panels, our results show detectable association between Orai1

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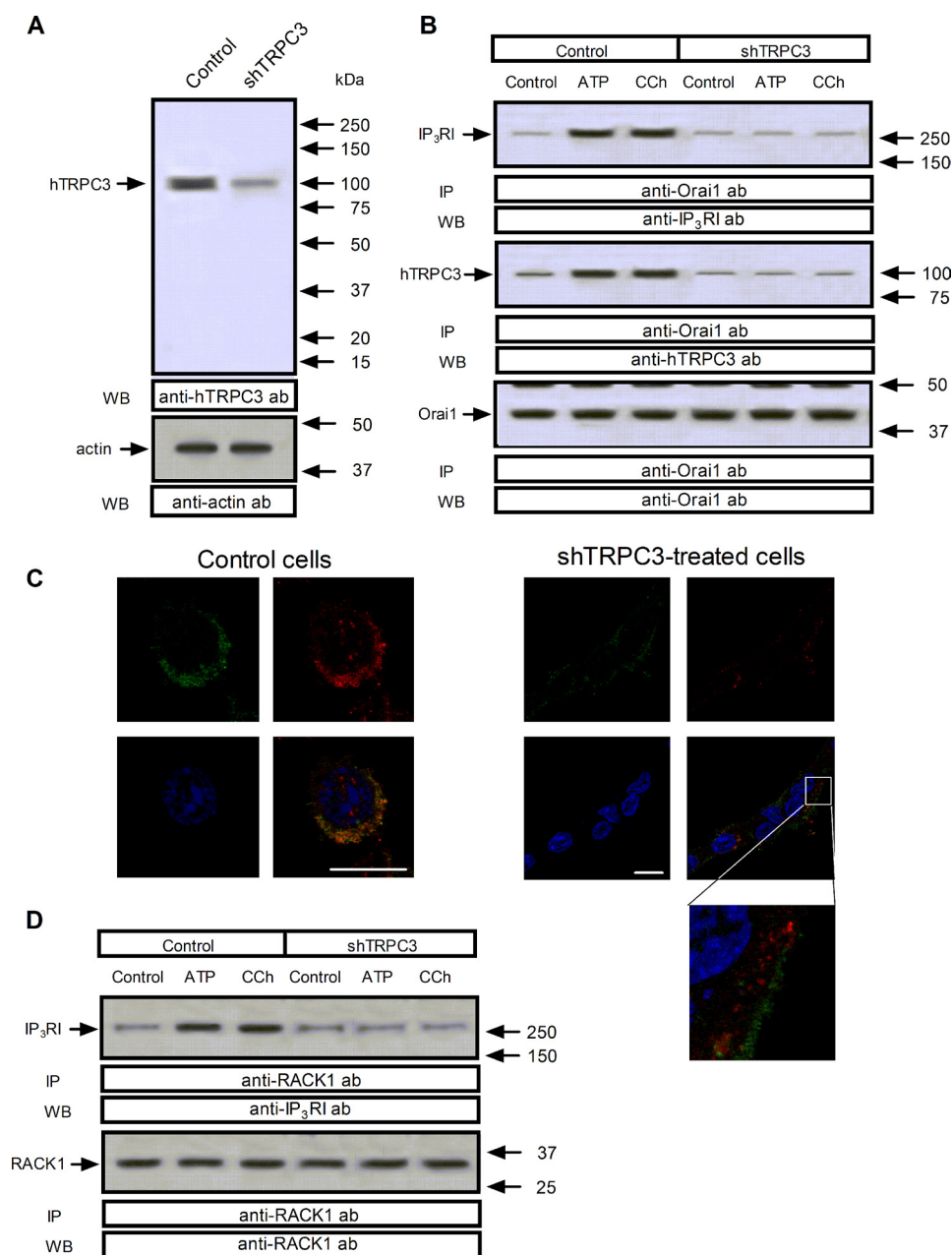


FIGURE 3. Role of TRPC3 in the association between IP₃RI and Orai1 or RACK1 in HEK cells. HEK293 cells were transfected with shTRPC3 or control vector, as indicated, and used after 48 h for protein detection. *A*, Western blotting (WB) showing the expression levels of TRPC3, which was significantly reduced in lysates from cells transfected with shTRPC3. *Bottom panel*, Western blotting with anti-G actin antibody for protein loading control. *B* and *D*, HEK293 cells were transfected with shTRPC3 or control vector, as indicated, and then stimulated for 3 min with 10 μ M ATP or 100 μ M CCh or left untreated and lysed. Samples were immunoprecipitated (IP) with anti-Orai1 antibody (*B*) or anti-RACK1 antibody (*D*) and immunoprecipitates were analyzed by Western blotting (WB) using anti-type I IP₃R (*B* and *D*, upper panels) or anti-TRPC3 antibodies (*B*, middle panel) and reprobbed with either anti-Orai1 antibody (*B*, lower panel) or anti-RACK1 antibody (*D*, lower panel) as described under "Experimental Procedures." The panels show results from one experiment representative of three to five. *C*, confocal images of TG-treated HEK293 cells (*left panels*) or TG-treated HEK293 cells transfected with shTRPC3 (*right panels*) immunostained with anti-Orai1 and anti-IP₃RI antibodies followed by Alexa Fluor 488- and 568-conjugated secondary antibodies, respectively, or DAPI. An overlay of the three images is depicted on the *bottom right-hand image*. Cells were stimulated with 1 μ M TG in medium containing 1 mM Ca²⁺. Scale bar, 10 μ m. Inset depicts a zoom of a representative area.

and type I IP₃R in resting HEK293 and HeLa cells. Interestingly, we have found that treatment with TG in a medium containing 1 mM Ca²⁺ enhanced the co-immunoprecipitation between Orai1 and the type I IP₃R in HEK293 and HeLa cells by 72 and 57%, respectively (Fig. 1, *A* and *B*, upper panels; $p < 0.05$; $n = 6$).

Western blotting with anti-Orai1 antibody confirmed a similar content of this protein in all lanes (Fig. 1, *A* and *B*, lower panels).

We have further investigated whether the interaction between these two Ca²⁺-handling proteins requires rises in [Ca²⁺]_i by using the intracellular Ca²⁺ chelator dimethyl-BAPTA, a procedure that abolishes the rise in [Ca²⁺]_i induced by TG (data not shown, but see Ref. 35). As shown in Fig. 1, *A* and *B*, in a Ca²⁺-free medium (100 μ M EGTA was added) dimethyl-BAPTA loading reduced the interaction between Orai1 and the type I IP₃R in resting HEK293 cells and prevented the TG-stimulated increase in co-immunoprecipitation of both proteins in HEK293 and HeLa cells. These findings suggest that TG enhances Ca²⁺-dependent association between Orai1 and type I IP₃R.

Next, we explored co-localization between Orai1 and type I IP₃R by immunofluorescence using specific anti-Orai1 and anti-type I IP₃R antibodies followed by Alexa Fluor 488- or 568-conjugated secondary antibodies both in control cells and cells stimulated with TG. As shown in Fig. 1, *C* and *D*, and previously described (35), Orai1 was distributed in the cell periphery (plasma membrane) but there is also detectable staining in the cytoplasmic area, whereas type I IP₃R shows a cytoplasmic distribution in resting HEK293 cells. Stimulation with TG for 3 min resulted in re-distribution of type I IP₃R in the cell periphery and enhanced co-localization (Fig. 1, *C* and *D*; the value of overlap coefficient according to Manders *et al.* (36) increased following stimulation with TG from 0.484 in resting cells to 0.723 in TG-treated cells).

Association of Type I IP₃ Receptor with Orai1, TRPC3, RACK1, and STIM1—Studies in HEK293 cells have reported that IP₃Rs, RACK1, and TRPC3 form a dynamically regulated complex that is important for agonist-induced Ca²⁺ mobilization (33, 41). Hence we have investigated the association of the type I IP₃R with TRPC3 and RACK1, as well as with STIM1, which associates with Orai1 during SOCE (10, 42, 43). As shown in Fig. 2, Orai1, TRPC3, RACK1, and STIM1 were

detected in type I IP₃R immunoprecipitates from resting cells. Association between these proteins with the type I IP₃R was enhanced by treatment with ATP and CCh by 69 ± 6 and $67 \pm 9\%$ for Orai1, 89 ± 4 and $95 \pm 10\%$ for TRPC3, 82 ± 7 and $89 \pm 6\%$ for RACK1, and 63 ± 4 and $59 \pm 3\%$ for STIM1, respectively ($p < 0.05$; $n = 6$). We have further investigated whether association of these proteins is related with the capacitative pathway for Ca²⁺ entry by inducing store depletion with TG in dimethyl-BAPTA-loaded cells. Dimethyl-BAPTA loading did not significantly modify the association between Orai1, TRPC3, RACK1, and STIM1 and slightly reduced the association of Orai1 with type I IP₃R in non-stimulated cells (data not shown). As shown in Fig. 2, in BAPTA-loaded cells TG was unable to induce association between type I IP₃R and Orai1, TRPC3, RACK1, or STIM1.

Role of TRPC3 in the Association between the Type I IP₃ Receptor and Orai1 and RACK1 Proteins—HEK293 cells have been reported to endogenously express several TRPC proteins, including TRPC3 (43, 44). In these cells, Orai1 has been demonstrated to physically interact with the N and C termini of the TRPC3 proteins (43). Recently, TRPC3 has been reported to regulate agonist-stimulated Ca²⁺ release by mediating interaction between IP₃R and RACK1 (33). Hence, we investigated the possibility that TRPC3 mediates interaction between Orai1 and type I IP₃R by using TRPC3 expression silencing.

As shown in Fig. 3A, *top panel*, shTRPC3 reduced significantly the amount of TRPC3 detected in HEK293 cells as compared with controls. Western blotting with anti-actin antibody revealed a similar amount of protein in all lanes (Fig. 3A, *bottom panel*). As reported above, treatment with ATP (10 μM) or CCh (100 μM) significantly increased the association between Orai1 and type I IP₃R by 70 ± 5 and $68 \pm 6\%$, respectively, and also between Orai1 and TRPC3 by 64 ± 5 and $68 \pm 4\%$, respectively (Fig. 3B, *lanes 1–3*; $p < 0.05$; $n = 5$). We found that shTRPC3 abolishes ATP- and CCh-mediated association between Orai1 and the type I IP₃R or TRPC3 (Fig. 3B, *top and middle panel*; $p < 0.05$; $n = 6$). Western blotting with anti-Orai1 antibody confirmed a similar content of this protein in all lanes (Fig. 3B, *lower panel*).

We have analyzed co-localization between Orai1 and type I IP₃R in shTRPC3-treated cells by immunofluorescence in cells stimulated with CCh. As shown in Fig. 3C, stimulation with CCh for 3 min resulted in redistribution of the type I IP₃R in the cell periphery and enhanced co-localization, which was impaired in shTRPC3-treated cells (the value of overlap coefficient according to Manders *et al.* (36) decreased in shTRPC3-treated cells stimulated with CCh from 0.795 in control cells to 0.184 in shTRPC3-treated cells).

We further explored the effect of shTRPC3 on interaction between type I IP₃R and RACK1. As shown in Figs. 2 and 3D and described above, treatment with ATP or CCh enhanced the association between the type I IP₃R and RACK1. Our results indicate that shTRPC3 abolishes ATP- and CCh-stimulated association between these proteins (Fig. 3D, *top panel*; $p < 0.05$; $n = 4$). Western blotting with anti-RACK1 antibody confirmed a similar content of this protein in all lanes (Fig. 3D, *lower panel*).

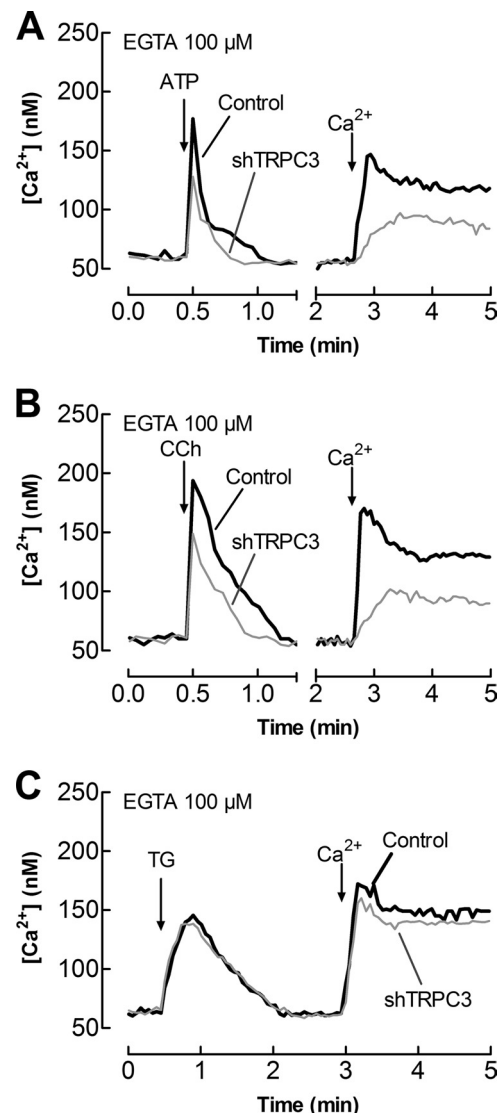


FIGURE 4. TRPC3 modulates Ca²⁺ mobilization. HEK293 cells were transfected with shTRPC3 (gray traces) or control vector (black traces) and used after 48 h for Ca²⁺ measurements. Cells were loaded with fura-2 and stimulated with 10 μM ATP (A), 100 μM CCh (B), or 1 μM TG (C), in a Ca²⁺-free medium (100 μM EGTA was added), followed by addition of CaCl₂ (final concentration 1 mM) to initiate Ca²⁺ entry. Traces are representative of five to 10 independent experiments.

TRPC3 Modulates Agonist-evoked Intracellular Ca²⁺ Release—Because silencing TRPC3 expression was effective preventing the association between Orai1 and type I IP₃R in HEK293 cells, we investigated the functional relevance of this interaction in agonist-evoked Ca²⁺ mobilization. Treatment of HEK293 cells with 10 μM ATP or 100 μM CCh induced a rapid and transient increase in [Ca²⁺]_i due to release of Ca²⁺ from the intracellular stores (Fig. 4). As shown in Fig. 4, A and B, shTRPC3 significantly decreased ATP- and CCh-induced intracellular Ca²⁺ release by 47 ± 9 and $39 \pm 7\%$ as compared with cells transfected with control vector ($p < 0.05$; $n = 5$). The effect observed is unlikely due to a different amount of Ca²⁺ accumulated in the intracellular stores because Ca²⁺ release evoked by TG was unaffected in cells treated with shTRPC3 (Fig. 4C). In addition, we found that TRPC3 expression silencing significantly attenuated ATP- and CCh-induced Ca²⁺ entry by 49 ± 9 and $51 \pm$

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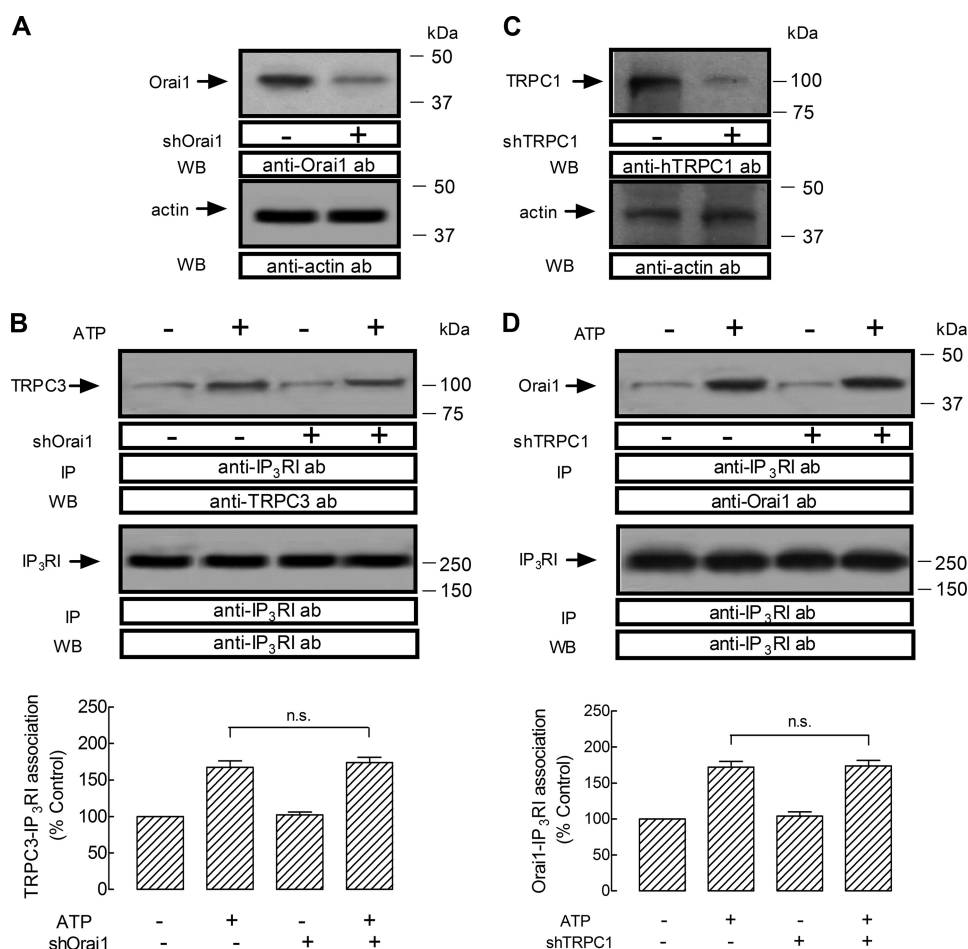


FIGURE 5. Role of Orai1 in the interaction TRPC3-type I IP₃R and lack of involvement of TRPC1 on the Orai1-type I IP₃R association. A and B, HEK293 cells were transfected with shOrai1 or control vector, as indicated, and used after 48 h for protein detection. A, Western blot (WB) showing the expression levels of Orai1, which was significantly reduced in lysates from cells transfected with shOrai1. *Bottom panel*, Western blotting with anti-G actin antibody for protein loading control. B, HEK293 cells were transfected with shOrai1 or control vector, as indicated, and then stimulated for 3 min with 10 μ M ATP or left untreated and lysed. Samples were immunoprecipitated (IP) with anti-type I IP₃R antibody and immunoprecipitates were analyzed by Western blotting using anti-TRPC3 antibody (*upper panel*) and reprobbed with anti-type I IP₃R antibody (*lower panel*) as described under "Experimental Procedures." The panels show results from one experiment representative of five. C and D, HEK293 cells were transfected with shTRPC1 or control vector, as indicated, and used after 48 h for protein detection. C, Western blot showing the expression levels of TRPC1, which was significantly reduced in lysates from cells transfected with shTRPC1. *Bottom panel*, Western blot with anti-G actin antibody for protein loading control. D, HEK293 cells were transfected with shTRPC1 or control vector, as indicated, and then stimulated for 3 min with 10 μ M ATP or left untreated and lysed. Samples were immunoprecipitated with anti-type I IP₃R antibody and immunoprecipitates were analyzed by Western blotting using anti-Orai1 antibody (*upper panel*) and reprobbed with anti-type I IP₃R antibody (*lower panel*) as described under "Experimental Procedures." The panels show results from one experiment representative of five. n.s., not significant; error bars, S.E.

7%, respectively; however, TRPC3 silencing did not significantly modify TG-evoked Ca²⁺ entry (Fig. 4, A–C; $p < 0.05$; $n = 10$).

We further investigated whether Orai1 is essential for the interaction between TRPC3 and type I IP₃R by Orai1 expression silencing. As shown in Fig. 5A, *top panel*, shOrai1 significantly reduced the amount of Orai1 detected in HEK293 cells. Western blotting with anti-actin antibody revealed a similar amount of protein in all lanes (Fig. 5A, *bottom panel*). Our results indicate that shOrai1 did not affect resting or the ATP-evoked increase in TRPC3-type I IP₃R association (Fig. 5B).

Because TRPC3 plays a crucial role in interaction between Orai1 and type I IP₃R, we investigated whether other TRPCs, such as TRPC1, would play such a role in this interaction. Our

results indicate that TRPC1 expression silencing by using shTRPC1, which reduces TRPC1 expression as compared with control cells (Fig. 5C), was without effect on resting or ATP-induced association between Orai1 and type I IP₃R (Fig. 5D). This finding indicates that TRPC1 is not involved in association between Orai1 and type I IP₃R, and suggest a specific role for TRPC3 in mediation of this complex.

Interaction of Orai1 with Types II and III IP₃ Receptors—To investigate whether the role of TRPC3 is specific for interaction between Orai1 and type I IP₃R and does not involve other IP₃R isoforms, we performed a series of experiments to assess the possible association of Orai1 with types II and III IP₃R in HEK293 cells. After immunoprecipitation with anti-Orai1 antibody, Western blotting revealed the presence of type II IP₃R in samples from TG-treated cells and also a faint immunoreactive band over 250 kDa was detected in resting cells (Fig. 6A, *upper panel*, $n = 6$). Similar results were observed in HeLa cells (Fig. 6C, *upper panel*), thus suggesting that this might be a general event in human cells. TG-induced coupling between Orai1 and type II IP₃R was independent of elevations in [Ca²⁺]_i because it was also observed, without significant decrement, in dimethyl-BAPTA-loaded cells. Western blotting with anti-Orai1 antibody confirmed a similar content of this protein in all lanes (Fig. 6, A and C, *lower panels*). By contrast, although we observed detectable association between Orai1

and the type III IP₃R at resting conditions both in HEK293 (Fig. 6B, *upper panel*) and HeLa (Fig. 6D, *upper panel*), we were unable to detect a significant increase in interaction between Orai1 and type III IP₃R upon stimulation with TG in these cells (Fig. 6, B and D), even though we used the same experimental procedures followed to detect coupling between Orai1 and types I or II IP₃R. It is noteworthy to mention that dimethyl-BAPTA loading reduced basal association between Orai1 and type III IP₃R in HEK293 cells (Fig. 6B, *upper panel*), as reported above for the type I IP₃R (Fig. 1A, *upper panel*). These findings indicate that although there is a small interaction between Orai1 and the three IP₃R isoforms, agonists only enhanced the interaction between Orai1 and types I and II IP₃R.

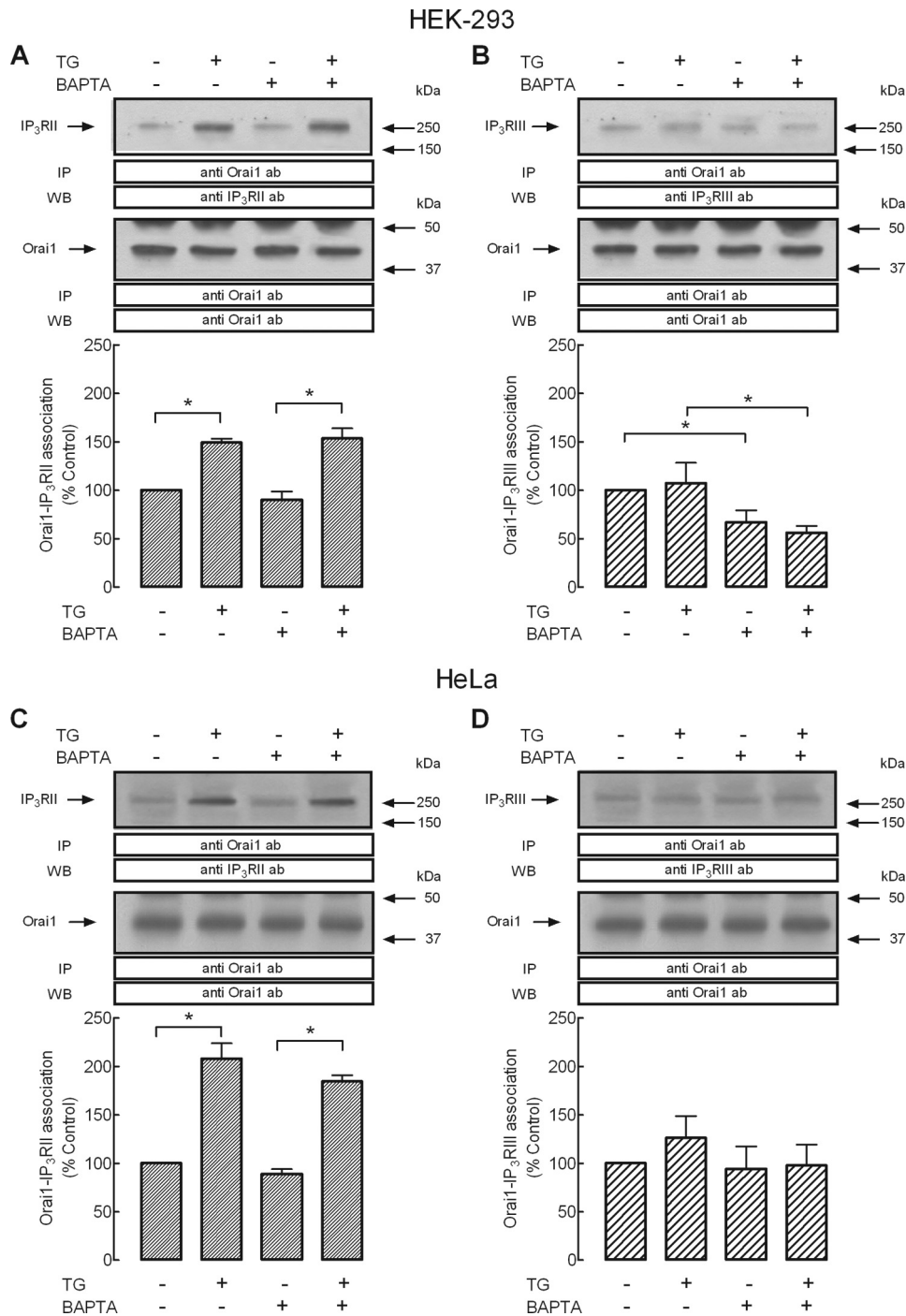


FIGURE 6. Interaction of Orai1 with types II and III IP₃R in HEK293 and HeLa cells. HEK and HeLa cells were either loaded with dimethyl-BAPTA or left untreated, as indicated, and then suspended in medium containing 1 mM Ca²⁺ or Ca²⁺-free medium (BAPTA-loaded cells, 100 μM EGTA was added). Cells were then stimulated with TG (1 μM) for 3 min or left untreated and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-Orai1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting (WB) with specific anti-IP₃R/III (A and C) or anti-IP₃R/III (B and D) antibodies. Membranes were reprobbed with the anti-Orai1 antibody for protein loading control. The panel shows results from one experiment representative of five. Molecular masses indicated on the right were determined using molecular mass markers run in the same gel. Histograms represent the quantification of Orai1-IP₃R/III or Orai1-IP₃R/III association under different experimental conditions. Results are expressed as mean ± S.E. and presented as percentage of control (non-stimulated cells not loaded with dimethyl-BAPTA). *, *p* < 0.05. Error bars, S.E.

Subsequently, we investigated whether TRPC3 expression silencing impairs the association between Orai1 and type II IP₃R. We observed that treatment with 10 μM ATP resulted in a

significant increase in association between Orai1 and type II IP₃R (Fig. 7, upper panel, lanes 1 and 2; *p* < 0.05). TRPC3 expression silencing had a negligible effect, if any, on resting or a ATP-evoked increase in Orai1-type II IP₃R association (Fig. 7, upper panel, lanes 3 and 4; *p* < 0.05). Western blotting with anti-type II IP₃R confirmed a similar content of this protein in all lanes (Fig. 7, lower panels). Therefore, our results indicate that the effect of TRPC3 silencing on agonist-induced Ca²⁺ release is specific for interaction between Orai1 and type I IP₃R.

DISCUSSION

TRPC3 proteins are among the first mammalian homologs of *Drosophila* TRP identified. The ability of TRPC3 to associate with a number of partner proteins has been reported to enable TRPC3 to participate in a variety of signals and transduction mechanisms (45). For instance, TRPC3 forming channels have been shown to be activated by diacylglycerol, although there is evidence suggesting that these channels can also be gated by IP₃R through a conformational coupling mechanism (46, 47). Moreover, evidence suggests that IP₃R are involved in translocation of TRPC3 proteins to the cell surface in response to agonist stimulation (48, 49). Reciprocally, recent studies have reported that TRPC3 is involved in modulation of Ca²⁺ release from the intracellular stores in different cell types, such as skeletal muscle cells (50) or HEK293 cells (33). The latter has been shown to be mediated by formation of a ternary complex, TRPC3-RACK1-IP₃R, although the nature of the IP₃R isoform involved has not yet been identified (33).

Our results report interaction between the type I IP₃R and Ca²⁺-handling proteins Orai1, STIM1, TRPC3, and RACK1. This protein complex is dynamically regulated by agonists, such as ATP and CCh, and plays a relevant role in Ca²⁺ release from intracellular stores and also Ca²⁺ entry. These findings are based on the effect of TRPC3 expression

Interaction between Orai1 and IP₃ Receptors

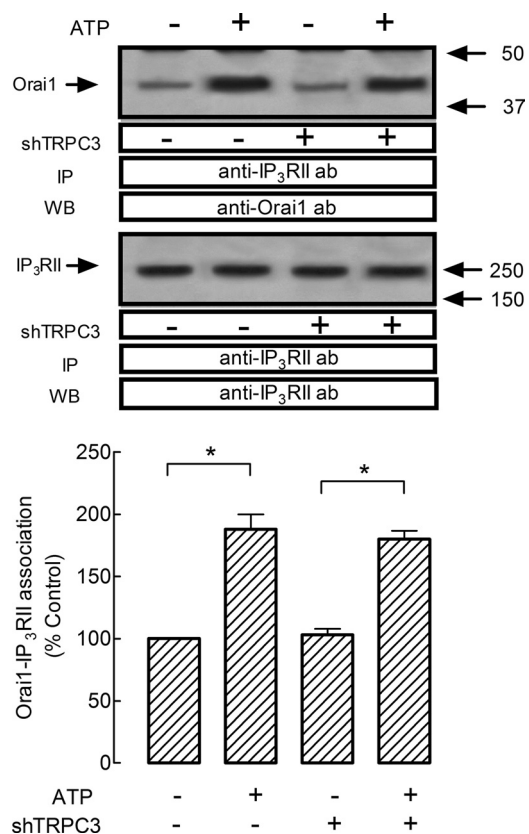


FIGURE 7. TRPC3 silencing does not alter the interaction between Orai1 and IP₃RII in HEK cells. HEK293 cells were transfected with shTRPC3 or control vector, as indicated, and used after 48 h for protein detection. Cells were stimulated for 3 min with 10 μ M ATP or left untreated and lysed. Samples were immunoprecipitated (IP) with anti-type II IP₃R antibody and immunoprecipitates were analyzed by Western blotting (WB) using anti-Orai1 antibody (upper panel) and reprobbed with anti-type II IP₃R antibody (lower panel) as described under "Experimental Procedures." The panels show results from one experiment representative of five. Histograms represent the quantification of Orai1-IP₃RII association under different experimental conditions. Results are expressed as mean \pm S.E. of six independent experiments and presented as percentage of control (cells transfected with control vector not treated with ATP). *, $p < 0.05$ compared to resting cells. Error bars, S.E.

silencing, which impairs association between type I IP₃R and the proteins Orai1 and RACK1, the latter is in agreement with previous studies (33). In addition, TRPC3 expression silencing inhibits agonist-evoked Ca²⁺ mobilization.

IP₃Rs have been reported to form macro signal complexes and function as a point of convergence for signaling cascades. A number of studies have reported the association between IP₃Rs and the scaffold protein RACK1 (41, 51, 52), which plays a crucial role in agonist-evoked Ca²⁺ release from intracellular stores, thus suggesting that RACK1 is a physiologic regulator of agonist-induced Ca²⁺ release (41).

We have found that cell treatment with shTRPC3 has no detectable effect on TG-induced Ca²⁺ release and entry; this finding, together with the observation that TG was unable to induce association between type I IP₃R and proteins Orai1, STIM1, TRPC3, and RACK1 in BAPTA-loaded cells, suggests that TRPC3 plays a crucial role in agonist-induced Ca²⁺ entry but not in SOCE in these cells. Our results indicate that functional Orai1 is not required for the TRPC3-type I IP₃R association, which further supports that TRPC3 plays a central role in the Orai1-TRPC3-type I IP₃R complex.

A major finding of this study is that TRPC3 mediates the interaction between Orai1 and type I IP₃R, whereas we suggest that it does not mediate the association between Orai1 and type II IP₃R. We show that disrupting TRPC3-Orai1 association by TRPC3 expression silencing impairs agonist-induced association between Orai1 and the type I IP₃R and results in significant inhibition of agonist-induced Ca²⁺ release and entry. By contrast, we have found that other TRPC proteins, such as TRPC1, are not required for the Orai1-type I IP₃R interaction. Our findings are in agreement with previous observations suggesting that TRPC3 mediates the interaction between IP₃R and RACK1, which has been reported to enhance IP₃-binding affinity and augment Ca²⁺ release (41). Recent studies have reported that impairment of TRPC3-RACK1 association by expressing mut-C3 or knocking down endogenous TRPC3 leads to attenuation of IP₃-mediated Ca²⁺ release (33), which, together with our results, indicates that TRPC3 might be a point of convergence of distinct signals regulating the IP₃R function.

Our results are consistent with previous studies reporting that TRPC proteins are involved in a dynamic interaction with IP₃Rs and Ca²⁺ signaling protein complexes (53–55). In addition, TRPC3 has been reported to regulate type I ryanodine receptor in primary mouse skeletal muscle myoblasts/myotubes (56).

Cell stimulation by agonists has been reported to lead to dynamic changes in Ca²⁺ signaling proteins involving trafficking and activation of cation permeable channels, such as TRPC3 and Orai1 (35, 57, 58). There is a body of evidence suggesting that TRPC3 proteins and Orai1 are recruited into the plasma membrane in signaling complexes containing IP₃Rs and other Ca²⁺-handling proteins (33, 48, 54, 57, 58). Consistent with this, we found the association between type I IP₃R and Orai1, STIM1, TRPC3, and RACK1. In addition, impairment of the association of Orai1 with TRPC3 and the type I IP₃R results in attenuation of agonist-evoked Ca²⁺ entry. Our results provide evidence supporting that the association between the type I IP₃R and TRPC3, RACK1, Orai1, and STIM1 is not capacitative in nature because TG was unable to induce formation of this complex in the absence of rises in [Ca²⁺]_i. In addition, impairment of this complex by shTRPC3 was without effect on TG-evoked SOCE. The role of STIM1 in non-capacitative receptor-regulated Ca²⁺ entry pathways, such as Ca²⁺ entry via arachidonate-regulated calcium (ARC) channels (59, 60) has previously been demonstrated. Our results reporting the association of STIM1 with type I IP₃R upon stimulation with ATP or CCh also shows the involvement of STIM1 in ROCE, further suggesting that STIM1 might be a universal regulator of Ca²⁺ entry.

In conclusion, our results indicate that agonist stimulation leads to the formation of a protein complex involving Orai1, STIM1, TRPC3, RACK1, and type I IP₃R, as well as Ca²⁺ release from the intracellular stores and Ca²⁺ entry from the extracellular medium. Here we report for the first time that disruption of association between Orai1 and TRPC3 prevents agonist-induced association of Orai1 with the type I IP₃R, leading to attenuated agonist-induced Ca²⁺ release and Ca²⁺ entry. Together these findings suggest that TRPC3 mediates the func-

tional coupling between Orai1 and the type I IP₃R and, therefore, modulates agonist-stimulated Ca²⁺ mobilization.

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