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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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\(_3\)Rs) interact with plasma membrane TRPC channels; however, at present there is no evidence supporting the interaction between Orai proteins and IP\(_3\)Rs. Here we report that treatment with thapsigargin or celastrol impairs the interaction of Orai1 and type I IP\(_3\)R, thus suggesting that TRPC3 selectively mediates interaction between Orai1 and type I IP\(_3\)R. In addition, TRPC3 expression silencing prevented the interaction of Orai1 with TRPC3 and, more interestingly, the association of Orai1 with the type II IP\(_3\)R, but not with the type II IP\(_3\)R, thus suggesting that TRPC3 selectively mediates interaction between Orai1 and type I IP\(_3\)R. In conclusion, our results indicate that agonist-induced Ca\(^{2+}\) release and entry.

Cellular stimulation by agonists results in a rise in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), 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 [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\(_3\)Rs), releases Ca\(^{2+}\) from non-mitochondrial intracellular Ca\(^{2+}\) stores (3). In non-excitable 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 (STIM1) 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\(_3\)Rs and human TRP channels has been demonstrated by different approaches in several cell types, including human platelets endogenously expressing TRPC1 and IP\(_3\)Rs (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\(_3\)Rs 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 (\(I_{\text{min}}\)), 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\(_3\)R function by mediating interaction between IP\(_3\)R 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...
of the Ca\(^{2+}\) permeable channel Orai1 with the type I and II IP\(_3\) receptors. The former appears to be mediated by TRPC3 proteins, which also mediates their interaction with RACK1. This protein complex might play a functional role in agonist-induced Ca\(^{2+}\) mobilization.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, thapsigargin (TG), leupeptin, benzamidine, 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 IP\(_3\) receptor and anti-type II IP\(_3\) 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 IP\(_3\) 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% CO\(_2\). The experiments were performed in HEPES-buffered saline containing (in mM): 145 NaCl, 10 HEPES, 10 d-glucose, 5 KCl, 1 MgSO\(_4\), pH 7.45. For dimethyl-BAPTA loading, cells were incubated for 30 min at 37 °C with 10 \(\mu\)M dimethyl-BAPTA-AM. Cells were then collected by centrifugation and resuspended in HEPES-buffered saline.

Cell transfection with shTRPC3, shTRPC1, and shOrai1 was performed as described previously (33, 34) using...
shTRPC3, shTRPC1, and shOrai1 kindly provided by Dr. Ambudkar. For shTRPC3, the sense sequence was 5'-CACCGTATGGTCTCAAGTAACGATTTGACACCACCATC-3', and the antisense sequence was 5'-AAAAATGTTGGTCTGATTTCGTACCTCCATGGCACCACATCAC-3'. For the shRNA targeting human TRPC1, the sense sequence was 5'-CACGGGTTGACTTTTATGGTTTCGAACCATATAATAGTCACCC-3', and the antisense sequence was 5'-AAAAGGGTGACTTATATGATTTTCGAACCACATATAATAGTCACCC-3'. For the shOrai1, the sense sequence was 5'-CACCTACGTGGTAGCCATAAGCAGCTATCTTATGGCTAACCAGTGACTC-3', and the antisense sequence was 5'-AAAACCTTACAGCTATGGTTCGTTACATTACATCCACC-3'. These sequences were synthesized and hybridized as previously described (33, 34). Plasmids were used for silencing experiments at 1 µg/µl.

**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 µg/ml of leupeptin, and 10 mM benzamidine. 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, Staffs, 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.

**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⁺], 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⁺], for 3 min after the addition of the agonist (38). [Ca²⁺]i entry was estimated using the integral of the rise in [Ca²⁺], for 2.5 min after addition of CaCl₂ (39). [Ca²⁺]i entry was corrected by subtraction of the [Ca²⁺], 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 IP₃, 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⁺],. As depicted in Fig. 1, A and B, upper panels, our results show detectable association between Orai1
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 Ca\(^{2+}\)-handling proteins requires rises in [Ca\(^{2+}\)], by using the intracellular Ca\(^{2+}\) chelator dimethyl-BAPTA, a procedure that abolishes the rise in [Ca\(^{2+}\)], induced by TG (data not shown, but see Ref. 35). As shown in Fig. 1, A and B, in a Ca\(^{2+}\)-free medium (100 \(\mu\)M EGTA was added) dimethyl-BAPTA loading reduced the interaction between Orai1 and the type I IP\(_3\)R 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 Ca\(^{2+}\)-dependent association between Orai1 and type I IP\(_3\)R.

Next, we explored co-localization between Orai1 and type I IP\(_3\)R by immunofluorescence using specific anti-Orai1 and anti-type I IP\(_3\)R 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 IP\(_3\)R shows a cytoplasmic distribution in resting HEK293 cells. Stimulation with TG for 3 min resulted in re-distribution of type I IP\(_3\)R 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 IP\(_3\) Receptor with Orai1, TRPC3, RACK1, and STIM1—**Studies in HEK293 cells have reported that IP\(_3\)R, RACK1, and TRPC3 form a dynamically regulated complex that is important for agonist-induced Ca\(^{2+}\) mobilization (33, 41). Hence we have investigated the association of the type I IP\(_3\)R 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 ± 9% for Orai1, 89 ± 4 and 95 ± 10% for TRPC3, 82 ± 7 and 89 ± 6% for RACK1, and 63 ± 4 and 59 ± 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 ± 6%, respectively, and also between Orai1 and TRPC3 by 64 ± 5 and 68 ± 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).

**Interaction between Orai1 and IP₃ Receptors**

*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 ± 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 ±
Interaction between Orai1 and IP₃ Receptors

A

Orai1
shOrai1
WB
anti-Orai1 ab
actin

B

TRPC3
ATP
- + - +

IP
WB
anti-IP₃R ab
actin

C

TRPC1
ATP
- + - +

IP₃R
WB
anti-IP₃R ab
actin

D

Orai1
ATP
- + - +

WB
anti-actin ab

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-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 μM 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 reprobed 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-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 μM 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 reprobed with anti-type I IP₃R antibody (lower panel) as described under “Experimental Procedures.” The panels show results from one experiment representative of five.

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⁺], 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.
Subsequently, we investigated whether TRPC3 expression silencing impairs the association between Orai1 and type II IP$_3$R. We observed that treatment with 10 $\mu$M ATP resulted in a significant increase in association between Orai1 and type II IP$_3$R (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 IP$_3$R association (Fig. 7, upper panel, lanes 3 and 4; $p < 0.05$). Western blotting with anti-type II IP$_3$R 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 Ca$^{2+}$/H$^{+}$ release is specific for interaction between Orai1 and type I IP$_3$R.

**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 IP$_3$Rs through a conformational coupling mechanism (46, 47). Moreover, evidence suggests that IP$_3$Rs 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 Ca$^{2+}$/H$^{+}$ 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-IP$_3$R, although the nature of the IP$_3$R isoform involved has not yet been identified (33).

**FIGURE 6. Interaction of Orai1 with types II and III IP$_3$R in HEK293 and HeLa cells.** HEK and HeLa cells were either loaded with dimethyl-BAPTA or left treated, as indicated, and then suspended in medium containing 1 mM Ca$^{2+}$/H$^{+}$ or Ca$^{2+}$/H$^{+}$-free medium (BAPTA-loaded cells, 100 $\mu$M EGTA was added). Cells were then stimulated with TG (1 $\mu$M) 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-IP$_3$RII (A and C) or anti-IP$_3$RIII (B and D) antibodies. Membranes were reprobed 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-IP$_3$RII or Orai1-IP$_3$RIII 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.
A major finding of this study is that TRPC3 mediates the interaction between Orai1 and type I IP$_3$R, whereas we suggest that it does not mediate the association between Orai1 and type II IP$_3$R. We show that disrupting TRPC3-Orai1 association by TRPC3 expression silencing impairs agonist-induced association between Orai1 and the type I IP$_3$R and results in significant inhibition of agonist-induced Ca$^{2+}$ release and entry. By contrast, we have found that other TRPC proteins, such as TRPC1, are not required for the Orai1-type I IP$_3$R interaction. Our findings are in agreement with previous observations suggesting that TRPC3 mediates the interaction between IP$_3$R and TRPC3, RACK1, Orai1, and STIM1 (41). Recent studies have reported that impairment of TRPC3-RACK1 association by expressing mut-C3 or knocking down endogenous TRPC3 leads to attenuation of IP$_3$-mediated Ca$^{2+}$ release (33), which, together with our results, indicates that TRPC3 might be a point of convergence of distinct signals regulating the IP$_3$R function.

Our results are consistent with previous studies reporting that TRPC proteins are involved in a dynamic interaction with IP$_{3}$Rs and Ca$^{2+}$ 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 Ca$^{2+}$ 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 IP$_{3}$Rs and other Ca$^{2+}$-handling proteins (33, 48, 54, 57, 58). Consistent with this, we found the association between type I IP$_{3}$R and Orai1, STIM1, TRPC3, and RACK1. In addition, impairment of the association of Orai1 with TRPC3 and the type I IP$_{3}$R results in attenuation of agonist-evoked Ca$^{2+}$ entry. Our results provide evidence supporting that the association between the type I IP$_{3}$R 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 [Ca$^{2+}$]$_{i}$. In addition, impairment of this complex by shTRPC3 was without effect on TG-evoked SOCE. The role of STIM1 in non-capacitative receptor-regulated Ca$^{2+}$ entry pathways, such as Ca$^{2+}$ entry via arachidonate-regulated calcium (ARC) channels (59, 60) has previously been demonstrated. Our results reporting the association of STIM1 with type I IP$_{3}$R upon stimulation with ATP or CCh also shows the involvement of STIM1 in ROCE, further suggesting that STIM1 might be a universal regulator of Ca$^{2+}$ 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 IP$_{3}$R, as well as Ca$^{2+}$ release from the intracellular stores and Ca$^{2+}$ 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 IP$_{3}$R, leading to attenuated agonist-induced Ca$^{2+}$ release and Ca$^{2+}$ 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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