TRPC3 Controls Agonist-stimulated Intracellular Ca\(^{2+}\) Release by Mediating the Interaction between Inositol 1,4,5-Trisphosphate Receptor and RACK1*§

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Activation of TRPC3 channels is concurrent with inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R)-mediated intracellular Ca\(^{2+}\) release and associated with phosphatidylinositol 4,5-bisphosphate hydrolysis and recruitment to the plasma membrane. Here we report that interaction of TRPC3 with receptor for activated C-kinase-1 (RACK1) not only determines plasma membrane localization of the channel but also the interaction of IP\(_3\)R with RACK1 and IP\(_{3}\)-dependent intracellular Ca\(^{2+}\) release. We show that TRPC3 interacts with RACK1 via N-terminal residues Glu-232, Asp-233, Glu-240, and Glu-244. Carbachol (CCh) stimulation of HEK293 cells expressing wild type TRPC3 induced recruitment of a ternary TRPC3–RACK1–IP3R complex and increased surface expression of TRPC3 and Ca\(^{2+}\) entry. Mutation of the putative RACK1 binding sequence in TRPC3 disrupted plasma membrane localization of the channel. CCh-stimulated recruitment of TRPC3–RACK1–IP3R complex as well as increased surface expression of TRPC3 and receptor-operated Ca\(^{2+}\) entry were also attenuated. Importantly, CCh-induced intracellular Ca\(^{2+}\) release was significantly reduced as was RACK1–IP3R association without any change in thapsigargin-stimulated Ca\(^{2+}\) release and entry. Knockdown of endogenous TRPC3 also decreased RACK1–IP3R association and decreased CCh-stimulated Ca\(^{2+}\) entry. Furthermore, an oscillatory pattern of CCh-stimulated intracellular Ca\(^{2+}\) release was seen in these cells compared with the more sustained pattern seen in control cells. Similar oscillatory pattern of Ca\(^{2+}\) release was seen after CCh stimulation of cells expressing the TRPC3 mutant. Together these data demonstrate a novel role for TRPC3 in regulation of IP3R function. We suggest TRPC3 controls agonist-stimulated intracellular Ca\(^{2+}\) release by mediating interaction between IP3R and RACK1.

The ability of eukaryotic cells to respond to various stimuli through changes in intracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)) is important for many cellular processes. Such changes involve both intracellular Ca\(^{2+}\) release, primarily via inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP3R) as well as Ca\(^{2+}\) entry via store-operated and store-independent Ca\(^{2+}\) entry channels (1). Transient receptor potential canonical (TRPC) channels constitute a family of relatively nonselective divergent cation channels that are activated in response to agonist-stimulated PIP2 hydrolysis (2, 3). Of these, TRPC3 and TRPC6 are activated by diacylglycerol and thought to form store-independent Ca\(^{2+}\) channels, although TRPC3 forms store-operated channels under certain circumstances (4–6). Dynamic recruitment of a TRPC6–IP3R–Ca\(^{2+}\) signaling complex has been previously reported (7). Similarly, TRPC3 is also assembled in a multimeric complex with key Ca\(^{2+}\) signaling proteins including IP3R and is recruited to the plasma membrane in response to agonist-stimulated PIP2 hydrolysis (8–10). Interaction with IP3R has been suggested to be involved in agonist activation of TRPC3 (11, 12), although this has been questioned in several studies (5, 13). Furthermore, it has been reported that IP3R together with Homer is involved in translocation of the channel to the cell surface in response to stimulation by an agonist (12, 14).

IP3R responds to the second messenger IP3 as well as ambient Ca\(^{2+}\) to generate cytosolic Ca\(^{2+}\) signals that are involved in regulating a wide variety of physiological functions. The localization of IP3R to specific regions in the cell is now considered an important factor in the spatial regulation of Ca\(^{2+}\) release. The molecular mechanism responsible for spatial distribution/redistribution of IP3R in cells after stimulation remains to be elucidated. Exquisite temporal and spatial control of IP3R function is achieved by the ability of the channel to integrate signals from numerous proteins including regulatory proteins, such as kinases and phosphatases, as well as scaffolding proteins such as Homer and RACK1 (36). RACK1 serves a central role in critical cellular processes such as growth and transduction of plasma membrane signals to downstream effector proteins.

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3 The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\); IP3R, inositol 1,4,5-trisphosphate (IP3) receptor; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol trisphosphate; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; TRPC, transient receptor potential canonical; HA, hemagglutinin; HEK, human embryonic kidney cell; aa, amino acid(s); wt, wild type; CCh, carbachol; IB, immunoblot.
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(15–18). It has been suggested to act as a cog-wheel to scaffold and facilitate the interaction(s) between signaling proteins via its seven internal WD40 (Trp-Asp 40) repeats. RACK1 is ubiquitously expressed in the tissues of higher mammals and humans including brain, liver, and spleen and has been shown to interact with IP\(_3\)R as well as other Ca\(^{2+}\) signaling proteins, e.g. phospholipase C\(_\gamma\), protein kinase C, and Src protein tyrosine kinase (19, 20). RACK1-IP\(_3\)R interaction was shown to increase the affinity of IP\(_3\)R for IP\(_3\) and, therefore, be required for agonist-dependent intracellular Ca\(^{2+}\) release (21).

Here we report that RACK1 is also an accessory protein for TRPC3 and that interaction between these two proteins determines plasma membrane localization and function of TRPC3. Our data demonstrate that agonist stimulation of cells results in recruitment of a TRPC3-RACK1-IP\(_3\)R ternary complex that is critical for both internal Ca\(^{2+}\) release via IP\(_3\)R and Ca\(^{2+}\) entry via TRPC3. Importantly, we show that interaction of TRPC3 with RACK1 and its localization within the plasma membrane region is critical in the control of IP\(_3\)R function. We show that TRPC3-RACK1 interaction is required for maintenance of RACK1-IP\(_3\)R interaction. Disruption of RACK1-TRPC3 interaction either by directed mutagenesis of the interaction domain in TRPC3 or silencing TRPC3 expression resulted in loss of RACK1-IP\(_3\)R interaction as well as reduction in CCh-stimulated intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry. Interestingly, an oscillatory pattern of CCh-stimulated intracellular Ca\(^{2+}\) release was seen in these cells compared with the more sustained pattern seen in control cells. Together these data demonstrate a novel role for TRPC3 in regulation of agonist-stimulated intracellular Ca\(^{2+}\) release. We suggest TRPC3 controls IP\(_3\)R function by mediating interaction between IP\(_3\)R and RACK1.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Transfection, and Crude Membrane Preparation—Human embryonic kidney cell 293 (HEK293) cells culture, transfection, and lysis were performed as previously described (8–10). Cells were harvested and lysed. Crude membranes were prepared from cell lysates and stored at −80 °C until used.

Yeast Two-hybrid Assay—NTRPC3 (residues 1–341) or CTRPC3 (residues 642–842) was subcloned into pGBKTT7 (GAL4 DNA binding domain; Clontech (10)) and used to transform the yeast reporter strain AH 109 (Clontech). Cells were selected by growth on SD-trp form the yeast reporter strain AH 109 (Clontech). Cells were (GAL4 DNA binding domain; Clontech (10)) and used to transform. CTRPC3 (residues 642–842) was subcloned into pGBKTT7 until used. Single site-directed mutagenesis. Oligonucleotides were synthesized as required for the mutagenesis, and PCR was performed using the QuikChange mutagenesis kit (Stratagene). After PCR, the parental DNA template was removed by DpnI endonuclease digestion, and the remaining PCR product was transformed into E. coli XL1-Blue cells. Colonies were selected, and each mutation was confirmed by sequencing.

shRACK1 and shTRPC3 Constructs—We have selected RNA-interference-sensitive target for RACK1 from previously published sequences (38). Both sense (5′-caccgcatggaattcctgggtcggaaaACACCGGTTACCATGAC-3′) and antisense (5′-aaaaagcttgaatcctgggttcgAACACCGGTTACCATGAC-3′) sequences, which were targeted to 496–514 bases downstream to the start codon ATG of human RACK1, were synthesized by Integrated DNA Technologies. To enable directional cloning, both sense and antisense oligo had 4-nucleotide overhangs at 5′-end of the oligo. These two oligos were then hybridized in equal molar ratios and cloned into pENTR/U6 vector. For the shTRPC3, a short cDNA sequence located 19 bp from exon 5 (nucleotides 1767–1785 of TRPC3 mRNA) was used to make the construct. The sense sequence was 5′-caccgcatggaattcctgggtcggaaaACACCGGTTACCATGAC-3′, and the antisense sequence was 5′-aaaaagcttgaatcctgggttcgAACACCGGTTACCATGAC-3′. Both were complementary sequences with proper overhangs and also contained a short loop. These sequences were also synthesized and hybridized as described earlier for shRACK1. The shRACK1 and shTRPC3 were then confirmed by sequencing and used for silencing experiments at the concentration of 1 μg/μl.

Immunoprecipitation, Western Blotting and Confocal Microscopy—Membrane solubilization, immunoprecipitation, SDS-PAGE, Western blotting, and ECL detection were performed as described earlier (9, 10). Briefly, crude membranes were solubilized either with radioimmune precipitation assay buffer or with 1.5 mM octylglucoside plus 0.5 mM KI, and the solubilized fraction was collected by centrifugation, precleared, and then incubated with the required antibody at 4 °C. Immunocomplexes were pulled down with 30 μl of 30% protein A beads. Beads were then washed with buffer containing 500 mM NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 10% sucrose 1 μg/ml aprotinin, leupeptin, and pepstatin and treated with SDS solubilization buffer. SDS-PAGE and Western blotting were conducted as described before (8–10). Anti-HA and anti-TRPC3 were used at 1:1000 dilutions. The secondary antibodies and conditions for the detection were as described previously. For immunoblotting (IB), primary antibodies were used at 1:1000 dilution. In control blots chick IgY or rabbit or rat, IgG was used instead of the primary antibodies (proteins were not detected; data not shown). For immunofluorescence cells were fixed, permeabilized, and treated with the required primary antibody (1:100 or 1:200 dilution). Further, cells were washed and incubated with required fluorescently-labeled secondary antibody (1:100 dilution). Hippocampal neuronal cultures (from E18 rats) were kept in neurobasal medium (Invitrogen) supplemented with B27 for 3 weeks. Confocal microscopy was conducted as described previously (39) using a Leica confocal microscope.
Cell Surface Biotinylation—Confluent HEK293 cells stably expressing HA- or FLAG-TRPC3 were incubated for 3 min with or without CCh (100 μM) at 37 °C in Ca²⁺-free SES medium (145 mM NaCl, 5 mM CsCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH)), washed, and incubated for 20 min with 0.5 mg/ml Sulfo-NHS-Biotin (Pierce) on ice. The cells were washed with buffer containing 0.1M glycine and solubilized with 2 ml of radioimmune precipitation assay buffer. Biotinylated proteins were pulled down with NeutrAvidin-linked beads (Pierce). Bound fraction was washed and released with SDS-PAGE sample buffer and analyzed by Western blotting. Protein were visualized with either NeutrAvidin horseradish peroxidase conjugate (Pierce) or anti-FLAG M2-peroxidase conjugate (Sigma) or anti-HA antibody (Clontech).

[Ca²⁺]i Measurements—Fura2 fluorescence in single cells was measured as described earlier (39) using an Olympus IX 50 microscope (Olympus) with a CoolSnap HQ Camera (Photometrics) attached to a Polychrome IV (Till Photonics LLC). MetaFluor (Molecular Devices) was used to acquire images and process the data. Analog plots of the fluorescence ratio (340/380) in single cells are shown.

Electrophysiological Measurements—Cell-attached patch clamp measurements were performed as described previously (40). Briefly, coverslips were transferred to the recording chamber and kept in a Ringer’s solution (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH)). The patch pipette had resistances between 3 and 5 megohms after filling with the standard intracellular solution (145 mM cesium methane sulfonate, 8 mM NaCl, 10 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, pH 7.2 (CsOH)). External solutions were 145 mM NaCl, 5 mM CsCl, 1 mM MgCl₂, 10 mM CaCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH). Patch clamp experiments were performed in the tight-seal whole cell configuration at room temperature (22–25 °C) using Axonpatch 200B amplifier (Axon Instrument). Development of the current was assessed by measuring the current amplitudes at a potential of −70 mV, taken from high resolution currents in response to voltage ramps ranging from −90 to 90 mV over a period of 1 s for every 4 s and digitized at a rate of 1 KHz. A liquid-junction potential of less than 8 mV was not corrected, and capacitive currents and series resistance were determined and minimized. For analysis, the first ramp was used for leak subtraction of the subsequent current records. P-Clamp 9 (Axon Instrument) and Origin 6 (OriginLab) were used for analyses of data.

RESULTS

Interaction of TRPC3 with RACK1—A yeast two-hybrid screen of a HEK cDNA library using the N terminus (aa 1–341, NTRPC3 and CTRPC3, respectively) and RACK1. -L-T, SD-trp · leu · SD-T-H-A, SD-trp · leu · his · ade2 · L, leucine; H, histidine; T, tryptophan; A, adenine. B, co-localization of stably expressed HA-TRPC3 with endogenous RACK1 in HEK293 cells. C, co-localization of endogenous TRPC3 and RACK1 in cultured rat hippocampal neurons. Green signal, anti-TRPC3 antibody; red signal, anti-RACK1 antibody. Arrows show RACK1 and TRPC3 signals in the synaptic terminals (overlay images are in the right panels, yellow signal). Differential interference contrast (DIC) images are shown in the left panels. The lower panels show high resolution images. D, co-immunoprecipitation of HA-TRPC3 with endogenous RACK1 in HEK293 cells. E, immunoprecipitation of TRPC3 and RACK1 from rat brain extracts. Antibodies used for IP and IB are as indicated in D and E (control IPs using IgG or non-transfected cells do not give similar reactions; data not shown). Inputs shown here and in subsequent figures were 1/10–1/20 that of the protein used for IP.
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**FIGURE 2.** Effect of mutations in the putative RACK1 binding domain of TRPC3 on its trafficking and plasma membrane expression. A, yeast two-hybrid interactions between the different fragments of NTRPC3 and RACK1. B, schematic diagram showing the position of mutations in the N3 domain of NTRPC3. C, anti-HA antibody and FITC-conjugated secondary antibody were used to detect TRPC3 in HEK293 cells stably expressing HA-tagged TRPC3 (wt-C3, upper panels) and mutant TRPC3 (mut-C3, middle panels) and wt-C3 in cells transfected with shRACK1 (bottom panels). D, Western blots showing IP of wt-C3 and mut-C3 with RACK1 (input shows that expression level of the wt and mutant proteins are similar). Other conditions are similar to those described for Fig. 1E, reverse IP using anti-RACK1 antibody. Immunoblotting with anti-HA to detect TRPC3 (upper band running below the 97-kDa marker). The blot on the right shows surface biotinylation of HA-TRPC3 (see "Experimental Procedures" for details). Surface expression of PMCA was not changed (data not shown). Input levels of TRPC3 in non-biotinylated and biotinylated samples were similar when probed with anti-HA (see the input samples in D; note that the lysate from the biotinylated cells was used for the IPs shown in D and E). F, Ca\(^{2+}\) influx was measured in 1-oleoyl-2-acetylgllycerol (OAG)-stimulated wt-C3 and mut-C3 cells as described under "Experimental Procedures".

We assessed the relevance of these acidic aa residues in the N-terminal region of TRPC3 either by deleting this domain (aa221–342, ΔN3-C3) or by site-directed mutagenesis of specific residues (Glu-232, Asp-233, Glu-240, and Glu-244 to Ala, mut-C3, Fig. 2B). In general, expression of both these constructs yielded similar results, but whereas the expression level of mut-C3 was similar to wt-C3, expression of the deletion mutant was lower (Fig. 2D, data with the deletion mutant was not shown). All the studies discussed below have been done with mut-C3 either stably or transiently expressed in HEK293 cells (both conditions yielded similar data). Mutating the putative RACK1-binding sequence resulted in disruption of the cellular interaction between HA-TRPC3 or IP\(_{\gamma}\)R co-immunoprecipitate with HA-TRPC3 (IP done using anti-HA antibody, transient, or stable expression of either HA-TRPC3 or FLAG-TRPC3 gave similar results). Note that G\(_{\gamma}\)y, which is similar to RACK1, was not detected in this IP fraction. Similarly, endogenous TRPC3 from rat brain co-immunoprecipitated with endogenous RACK1 and protein kinase C\(\beta\)2 (Fig. 1E, protein kinase C\(\beta\)2 is a well-established RACK1-interacting protein and was used as a positive control). We have not assessed the role of protein kinase C\(\beta\)2 in TRPC3 or IP\(_{\gamma}\)R function in the present study, although protein kinase C has been previously shown to affect TRPC3 activity (22). In reverse IP, RACK1 was detected in the immunoprecipitate of HA-TRPC3. Additionally, rabbit IgG did not pull down HA-TRPC3 or FLAG-TRPC3 (data not shown, also see Refs. 8 and 10).

TRPC3 Targeting to the Plasma Membrane Depends on Its Ability to Interact with RACK1—The RACK1 binding domain in TRPC3 was mapped by using yeast two-hybrid analysis (Fig. 2A). Three overlapping regions of NTRPC3 terminus were used as bait with the full-length RACK1 construct. The N3 region spanning aa 221–342 was found to interact with RACK1. We used the strategy employed previously to identify RACK1 binding domains in protein kinase C\(\beta\)2, RGS11, and PDE4D5 (18, 19, 23, 24) to examine possible RACK1 binding sequences in N3-TRPC3. This approach revealed a consensus acidic repeat motif (Glu-232, Asp-233, Glu-240, and Glu-244) which was designated as a putative RACK1 binding domain in TRPC3 (supplemental Fig. 1).
FIGURE 3. Effect of disruption of TRPC3-RACK1 binding on TRPC3 function. A, i, Ca^{2+} influx stimulated by CCh in mut-C3- and wt-C3-expressing cells. ii, average data for both Ca^{2+} release and influx was obtained by subtracting the resting fluorescence from the peak; asterisks denote values that are significantly different from that of the respective controls (p < 0.02, number of cells is indicated in each case). B, i, CCh stimulated inward current in wt-C3 and mut-C3 expressing cells. ii, i–v relations of the maximum current seen in i. Current amplitudes at −80 mV for wt-C3 was 6.5 ± 1.3 pA/picofarads (pF, n = 4), whereas for mut-C3 was 3.7 ± 0.9 pA/picofarads (n = 5). This difference was statistically significant (p < 0.01). C, thapsigargin-stimulated internal Ca^{2+} release and Ca^{2+} entry in cells expressing wt-C3 and mut-C3. D, effect of thimerosal in cells expressing wt-C3, ΔN3-C3, and mut-C3 (cells were treated in Ca^{2+}-free medium to detect internal Ca^{2+} release). CCh-induced release in the same cells is shown for comparison. Average data for the release was obtained by subtracting the resting fluorescence from the peak. E, effect of 1 μM Gd^{3+} on CCh-stimulated Ca^{2+} entry cells expressing wt-C3 (i) and mut-C3 (ii).
localization of TRPC3. Although wt-C3 was localized in the plasma membrane region of HEK cells (Fig. 2C, top panels), the mutant was localized intracellularly (Fig. 2C, middle panels). Consistent with this, knockdown of endogenous RACK1 also resulted in a disruption of TRPC3 localization in cells (Fig. 2C, lower panels). It was interesting that co-expression of the wt-C3 with the mutant partially rescued plasma membrane targeting of mut-C3 (supplemental Fig. 2), which is likely due to hetero-meric interactions between the proteins. Consequences of the mutation in the RACK1 binding domain of TRPC3 were further examined by co-immunoprecipitation experiments. Immunoprecipitation of RACK1 with mut-C3 was lower (Fig. 2D, IP using anti-HA antibody) than with wt-C3. Conversely, less mut-C3 was co-immunoprecipitated with RACK1 when anti-RACK1 antibody was used for the IP (Fig. 2E, left blot). Importantly, disrupted trafficking of mut-C3 was reflected by a decrease in its surface expression. Less mut-C3 was detected in the biotinylated fraction compared with wt-C3 (Fig. 2E, right blot). Consistent with the decrease in plasma membrane expression of mut-C3, 1-oleoyl-2-acetylglycerol-stimulated Ca\(^{2+}\) entry was substantially decreased >70\% in cells expressing the mutant channel compared with those expressing wt-C3 (Fig. 2F).

Together, these data demonstrate that binding to RACK1 is important for trafficking TRPC3 to the plasma membrane.

Effect of Disruption of TRPC3-RACK1 Binding on Agonist Regulation of TRPC3—Muscarinic receptor stimulation has been widely used to activate TRPC3 in HEK293 cells (10, 12, 22, 25). Fig. 3A shows that CCh-stimulated Ca\(^{2+}\) entry as well as intracellular Ca\(^{2+}\) release were significantly decreased by >70\% in cells expressing mut-C3 compared with those expressing wt-C3. Thapsigargin-stimulated internal Ca\(^{2+}\) release and Ca\(^{2+}\) entry were similar in the two groups of cells (Fig. 3A) demonstrating that disruption of RACK1-TRPC3 association does not affect endoplasmic reticulum calcium content or store-operated calcium entry per se. CCh-stimulated non-selective cation current was also significantly reduced in cells expressing the mut-C3 when compared with wt-C3 (Fig. 3B, i and ii). Because CCh can activate store-operated as well as store-independent TRPC3 channels (4, 25), we further examined which component was affected when TRPC3-RACK1 interaction was impaired. Fig. 3Ei shows the effect of 1 \(\mu\)M Gd\(^{3+}\) on Ca\(^{2+}\) entry in cells expressing wt-C3. Although a portion of the Ca\(^{2+}\) entry was blocked by 1 \(\mu\)M Gd\(^{3+}\), there was a significant level of Gd\(^{3+}\)-insensitive Ca\(^{2+}\) entry due to store-
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**FIGURE 5. Effect of TRPC3 knockdown on CCh-induced intracellular Ca\textsuperscript{2+} release and RACK1-IP\textsubscript{3}R interaction.** HEK293 cells were transfected with shTRPC3 (red traces) or control vector (black traces) and used after 48 h for functional measurements or lysed for IP. A–C, average CCh-stimulated intracellular Ca\textsuperscript{2+} release (from 50 cells) was assessed by stimulation of cells with varying concentrations of CCh (1, 20, and 100 µM) in Ca\textsuperscript{2+}-free external medium. Representative traces from control and shTRPC3-treated cells are given below. con, control. In C, representative traces from mut-C3 cells were also shown in addition to those of control and shTRPC3-treated cells. Average decreases in peak Ca\textsuperscript{2+} increase are shown in D. Knockdown of TRPC3 did not change thapsigargin-induced Ca\textsuperscript{2+} release (data not shown). The inset in B shows the Western blot whereby expression levels of TRPC3 were significantly reduced in lysates from cells transfected with shTRPC3. E shows the effect of TRPC3 knockdown on co-immunoprecipitation of IP\textsubscript{3}R with RACK1. Lysates of cells transfected with shTRPC3 or control were treated with anti-RACK1 antibody to immunoprecipitate RACK1. Samples were resolved by SDS gels and then examined for levels of RACK1 (top blots) and IP\textsubscript{3}R (lower blots).

Independent entry by TRPC3. However, in cells expressing mut-C3, the latter component was minimal, and almost all of the CCh-stimulated Ca\textsuperscript{2+} appeared to be Gd\textsuperscript{3+}-sensitive (Fig. 3Eii). Thus, loss of binding to RACK1 appears to primarily impact store-independent TRPC3 channels. Further studies are required to exclude the possibility that store-dependent TRPC3 channels might also depend on the interaction with RACK1. However, as noted above, there is minimal difference between wt-C3 and mut-C3 cells in thapsigargin-stimulated Ca\textsuperscript{2+} entry.

That Ca\textsuperscript{2+} entry was decreased in cells expressing mut-C3 was not surprising as the channel was not trafficked properly to the plasma membrane and its surface expression was also decreased (see Fig. 2, C and E). However, the decrease in CCh-stimulated intracellular Ca\textsuperscript{2+} release in cells expressing mut-C3 was very intriguing (Fig. 3E, i and ii). To examine this further, we tested the effect of thimerosal in the two sets of cells. Thimerosal increases the affinity of IP\textsubscript{3}R for IP\textsubscript{3} and, thus, results in activation of IP\textsubscript{3}R in “resting” cells, i.e. without PIP\textsubscript{2} hydrolysis and increase in IP\textsubscript{3} (26). Fig. 3D shows that although thimerosal induced a rapid and large internal Ca\textsuperscript{2+} release in wt-C3 and control HEK293 cells (data with former is shown in the figure, comparable with that with CCh), the increase in [Ca\textsuperscript{2+}], was significantly decreased in cells expressing both the mut-C3 and ΔN3-C3 mutants (also see supplemental Fig. 2B).

Together the results in Fig. 3 demonstrate that disruption in RACK1-TRPC3 binding decreases TRPC3-mediated Ca\textsuperscript{2+} entry as well as IP\textsubscript{3}-dependent intracellular Ca\textsuperscript{2+} release.

**CCh-stimulated Recruitment of TRPC3-IP\textsubscript{3}R-RACK1 Complex and TRPC3 Translocation to the Plasma Membrane—** Treatment of cells with CCh induced an increase in the association of FLAG-tagged wt-C3 with RACK1 (Fig. 4A) and IP\textsubscript{3}R3 (Fig. 4Aii). The levels of FLAG-TRPC3 in both samples and in the IPs were similar (Fig. 4Aii). Similar results were obtained with HA-tagged wt-C3 (data not shown). Reverse IP of the same lysate using an anti-RACK1 antibody also demonstrated a similar increase in the association of wt-C3 (Fig. 4Aiv) and IP\textsubscript{3}R
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Thus, CCh stimulation results in recruitment of a TRPC3-RACK1-IP\(_3\)R complex. In the case of mut-C3, CCh did not induce an increase in its association with either RACK1 or IP\(_3\)R (Fig. 4B, i and ii). Reverse IP using the anti-RACK1 antibody also demonstrated reduced association of TRPC3 with RACK1 (Fig. 4B, iv and v). Note that the levels of wt-C3 and mut-C3 were similar (Fig. 4Biii). Although the decreased association of the mut-C3 with RACK1 and IP\(_3\)R can be explained by its altered trafficking, the decreased association of IP\(_3\)R with RACK1 (Fig. 4Bv) was quite surprising and suggests that disruption of the interaction between TRPC3 and RACK1 also affects the interaction between IP\(_3\)R and RACK1. CCh stimulation of the surface expression of TRPC3 was also attenuated by mutation of its RACK1 binding domain as compared with the regulated trafficking of wt-C3 (Fig. 4Cii, ii shows that surface expression of PMCA, as a control plasma membrane protein, was not altered). These data suggest that binding to RACK1 localizes TRPC3 in a domain from where it can be inserted into the plasma membrane in response to stimulation of PIP\(_2\) hydrolysis. Furthermore, this binding also determines the functional status of IP\(_3\)R.

Knockdown of TRPC3 Reduces CCh-stimulated Internal Ca\(^{2+}\) Release—The data presented above suggest that TRPC3 can modulate IP\(_3\)R function. Thus, we examined the effect of knocking down endogenous TRPC3 on CCh-stimulated intracellular Ca\(^{2+}\) release. The data in Fig. 5, A–D, show that shTRPC3 induced a decrease in intracellular Ca\(^{2+}\) release. The reduction in the average peak amplitude of the response was more pronounced at the lower agonist concentrations (Fig. 5D; % decrease in release was 25% at 100 \(\mu M\) CCh versus >50% at 1 \(\mu M\) CCh). However, the [Ca\(^{2+}\)]\(_i\) increase due to CCh-stimulated intracellular release in cells treated with shTRPC3 was more oscillatory than that in control cells (traces from three representative cells are shown for each CCh concentration). This change in the pattern of response was especially evident at the higher CCh concentration (>10 \(\mu M\)), where control cells display a more sustained pattern of intracellular Ca\(^{2+}\) release (Fig. 5, B and C). Typically, >80% of the cells displayed this change in the pattern (in each condition we examined the response of more than 200 cells). A similar pattern of oscillations was also observed in mut-C3 cells (Fig. 5C). As expected, CCh-stimulated Ca\(^{2+}\) entry was also significantly decreased in cells treated with shTRPC3 (data not shown). The inset in Fig. 5B shows the decrease in TRPC3 levels in shTRPC3-treated cells. Furthermore, lysates from control cells or cells transfected with shTRPC3 were used for immunoprecipitation using anti-RACK1 antibody. Co-immunoprecipitation of IP\(_3\)R with RACK1 was highly decreased in cells transfected with shTRPC3 (Fig. 5E, left blot). Thus, knockdown of TRPC3 decreased interaction of RACK1 and IP\(_3\)R as was seen in cells expressing TRPC3 with mutation in the RACK1 binding domain. This decreased RACK1-IP\(_3\)R association is consistent with the suggested role for RACK1 in modulating the affinity of IP\(_3\)R for IP\(_3\) and can account for the decreased IP\(_3\)R function seen in these cells. Together, these data suggest that TRPC3 mediates the interaction between IP\(_3\)R and RACK1 and, thus, controls IP\(_3\)R function after stimulation of cells. It is interesting that downregulation of TRPC3 expression was shown to decrease ryano-
folds to assemble a signaling complex centered on the IP$_3$R. Previous studies have suggested that TRPC3 channels are assembled in a signaling complex with IP$_3$Rs and other Ca$^{2+}$ signaling proteins (2, 8–10, 12, 30–33). It has also been suggested that dynamic changes within this complex after agonist stimulation of cells appear to be involved in trafficking and activation of TRPC3 channels. Furthermore, a role for IP$_3$R in activation and trafficking of TRPC3 channel has been suggested. However, to our knowledge a role for TRPC3 in IP$_3$R-dependent Ca$^{2+}$ release has not yet been reported. Our data reveal a novel possibility that the interaction between RACK1 and TRPC3 determines the interaction between IP$_3$R and RACK1 and, consequently, IP$_3$R function. It is interesting to note that TRPC3 has been recently reported to interact with RyR and regulate its function (27). Together with our findings, this reflects a critical intrinsic property of TRPC3 channels.

Although further studies will be required to elucidate the molecular basis for the formation and regulation of the TRPC3-RACK1-IP$_3$R complex, the present data demonstrate that the N terminus of TRPC3 binds to RACK1, whereas the C terminus of the channel binds to the N terminus of IP$_3$R. Both TRPC3 and RACK1 binding domains on the N terminus of IP$_3$R have been mapped previously (21, 34–36). TRPC3 binds to F2q (aa 669–692) and F2g (aa 751–821) in the IP$_3$R N terminus, and expression of IP$_3$R-Fq domain was shown to attenuate CCh-stimulated TRPC3 function (34). RACK1 also binds to the N terminus of IP$_3$R in regions spanning aa 90–110 and 580–600 (21, 35, 36). Deletion of the first RACK1 binding domain on IP$_3$R decreased affinity of IP$_3$, whereas deleting the second domain completely inhibited IP$_3$R function (21). It is interesting to note that the first RACK1 binding domain overlaps with the suppressor region of IP$_3$R, whereas the second overlaps with the α region of IP$_3$R (aa 437–604) (35, 36). It has been suggested that the α region of IP$_3$R can bind to the suppressor region and, thus, impact binding of IP$_3$ to IP$_3$R. Therefore, modulation of the suppressor or ligand binding domains of IP$_3$R can have significant effects on its function, and this could be one mode by which RACK1 regulates IP$_3$R function. An important aspect of the interactions between RACK1, IP$_3$R, and TRPC3 is that both IP$_3$R and TRPC3 appear to interact with the third and fourth WD repeat regions of RACK1. Although Patterson et al. (21) mapped RACK1 aa 103–178 as the IP$_3$R binding domain, our data demonstrate that RACK1 aa 71–155 is a putative TRPC3 binding region. Thus, there appears to be an overlap between the IP$_3$R and TRPC3 binding regions on RACK1. How disruption of TRPC3 binding to RACK1 affects IP$_3$R binding is not yet known. A possible explanation is that loss of RACK1 binding results in the mislocalization of TRPC3 and prevents its trafficking to the plasma membrane region and interaction with the IP$_3$R. We hypothesize that loss of binding of TRPC3 leads to changes in the IP$_3$R such that its interaction with RACK1 is decreased. Therefore, TRPC3 appears to have a scaffolding role whereby its interaction with IP$_3$R facilitates IP$_3$R-RACK1 interaction. We propose that dynamic regulation of the TRPC3-RACK1-IP$_3$R complex after agonist stimulation not only determines activation of TRPC3-dependent Ca$^{2+}$ entry but also IP$_3$-mediated intracellular Ca$^{2+}$ release. The latter is possibly achieved by intramolecular rearrangement in IP$_3$R which fine-tunes its affinity for IP$_3$. Although there are no data available, it would be interesting to see if an increase in the recruitment of the TRPC3-RACK1-IP$_3$R complex is associated with an increase in the affinity of IP$_3$R for IP$_3$. Such regulation might allow a high level of IP$_3$R function to be maintained even after [Ca$^{2+}$], or IP$_3$ have decreased in agonist-stimulated cells.

In conclusion, we report that agonist stimulated PIP$_2$ hydrolysis leads to an increase in a TRPC3-IP$_3$R-RACK1 ternary complex together with activation of intracellular Ca$^{2+}$ release and TRPC3-mediated store-independent Ca$^{2+}$ entry. The latter involves increased surface expression of TRPC3, as shown here and by earlier studies. The main findings of this study are that loss of TRPC3-RACK1 binding (i) disrupts targeting of TRPC3 to the plasma membrane region of the cell and attenuates its surface expression after agonist stimulation, (ii) reduces recruitment of TRPC3-RACK1-IP$_3$R complex, and (iii) decreases IP$_3$R interaction with RACK1 and consequently IP$_3$-induced Ca$^{2+}$ release. Similarly, knockdown of TRPC3 also resulted in a decrease in CCh-stimulated intracellular Ca$^{2+}$ release as well as impaired RACK1-IP$_3$R interaction. As noted above, RACK1 has been previously reported to regulate IP$_3$R function by controlling its affinity for IP$_3$ (21, 36). Here we report for the first time that TRPC3 is also required for IP$_3$R function. We show that knockdown of TRPC3 or expression of TRPC3 mutants which lack RACK1 binding result in reduced RACK1-IP$_3$R interaction and in decreased CCh-stimulated intracellular Ca$^{2+}$ release. Furthermore, oscillatory rather than sustained [Ca$^{2+}$]$_i$ oscillations were induced by CCh stimulation in both these cases. Together these data suggest that TRPC3 mediates the interaction between IP$_3$R and RACK1 and, therefore, controls agonist-stimulated intracellular Ca$^{2+}$ release. IP$_3$R is a central and critical Ca$^{2+}$ signaling protein in cells. It is assembled in a complex with both plasma membrane, cytosolic, and endoplasmic reticulum proteins. IP$_3$R function is tightly controlled, and the channel is involved in regulating both physiological and cell death pathways. Studies over the past several years have demonstrated that IP$_3$Rs interact with plasma membrane TRPC channels (37) and, more recently, with other TRP channels such as PKD2 (41). Although many of these earlier studies propose a role for IP$_3$R in the regulation of TRPC channel function, to our knowledge the findings we have presented here provide the first demonstration that TRPC channels have a role in intracellular Ca$^{2+}$ release via IP$_3$R.

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