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Dynamic assembly of TRPC1/STIM1/Orai1 ternary complex is involved in store operated calcium influx: Evidence for similarities in SOC and CRAC channel components.

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Abstract

Store-operated calcium entry (SOCE) is a ubiquitous mechanism that is mediated by distinct store-operated Ca2+ (SOC) channels, ranging from the highly selective CRAC (calcium release-activated Ca2+) channel in rat basophilic leukemia and other hematopoietic cells to relatively Ca2+ selective or non-selective SOC channels in other cells. Although the exact composition of these channels is not yet established, TRPC1 contributes to SOC channels and regulation of physiological function of a variety of cell types. Recently, Orai1 and STIM1 have been suggested to be sufficient for generating CRAC channels. Here we show that Orai1 and STIM1 are also required for TRPC1-SOC channels. Knockdown of TRPC1, Orai1, or STIM1 attenuated while overexpression of TRPC1, but not Orai1 or STIM1, induced an increase in SOCE and ISOC in human salivary gland cells, HSG. All three proteins were co-localized in the plasma membrane region of cells and thapsigargin increased co-immunoprecipitation of TRPC1 with STIM1, and Orai1 in HSG as well as dispersed mouse submandibular gland cells. In aggregate, the data presented here reveal that all three proteins are essential for generation of ISOC in these cells and that dynamic assembly of TRPC1/STIM1/Orai1 ternary complex is involved in activation of SOC channel in response to internal Ca2+ store depletion. Thus, these data suggest a common molecular basis for SOC and CRAC channels.
Introduction

Store-operated Ca\(^{2+}\) entry (SOCE) is stimulated in response to depletion of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores (primarily the endoplasmic reticulum, ER) and is mediated via the activation of specific plasma membrane channels, termed store-operated calcium (SOC) channels. SOCE is ubiquitously expressed in all cell types and critically regulates a variety of cellular functions ranging from T-lymphocyte activation, smooth muscle contraction, platelet aggregation, fluid and protein secretion to regulation of cell growth and proliferation (1-3). Despite intense focus on SOCE over the past two decades neither the mechanism(s) by which the status of Ca\(^{2+}\) in the ER is transmitted to the PM, to activate or inactivate SOC channels, nor the molecular components of the channels have yet been identified. Interestingly, the characteristics of these channels in different cell types are quite distinct, suggesting diversity in the channel components (2,4-6). The first store-operated Ca\(^{2+}\) channel to be identified was the highly Ca\(^{2+}\)-selective, calcium release-activated calcium (CRAC) channel which is found in T-lymphocytes, RBL, and other hematopoietic cells (7,8). However, similar measurements in other cell types such as salivary gland, endothelial, and smooth muscle cells have demonstrated the presence of different types SOC channels, that range from non-selective to relatively Ca\(^{2+}\) selective (2,4-6,9). While the physiological significance of such diversity in SOC channels is not clear, it is important to consider whether all these channels are activated by the same signal generated in response to internal Ca\(^{2+}\) store depletion or whether internal Ca\(^{2+}\) store depletion induces multiple intracellular signals that act on different channels.

Members of the transient receptor potential canonical (TRPC) family of channels have been proposed as components of SOC channels (2-4,9-11). Although several TRPC members, e.g. TRPC4, TRPC3, TRPC7, have been reported to be activated by internal Ca\(^{2+}\) store depletion, the data are most consistent for TRPC1. This protein has been shown to be required for SOCE in several different cell types including salivary gland, HEK293, smooth muscle, endothelial, DT 40 cells, and platelets (4,9,12-15). These findings have been further substantiated by studies demonstrating that TRPC1 contributes to the Ca\(^{2+}\) permeability of SOC channels in several cell types, including salivary gland and smooth muscle cells (2,4,10,11,16,17). With the exception of a few studies which implicate TRPCs in CRAC function in lymphocytes (7,8,18) the molecular components of the CRAC channel are largely unknown. Recently two new proteins have emerged as candidate components of SOCE, STIM1 and Orai1. STIM1 is a single-transmembrane protein that has an unpaired EF-hand domain in the N-terminus which has been predicted to be localized in the ER lumen (19). Knockdown of STIM1 expression using siRNA significantly reduced SOCE in HEK293, SH-SY5Y, Jurkat T and HeLa cells (20,21). In contrast, over-expression of STIM1 only modestly enhanced SOCE in HEK293 cells (22). Additionally, STIM1 was relocalized into punctae in the subplasma membrane region following stimulation by thapsigargin (Tg) (20). The EF-hand domain of STIM1 has been suggested to
function as the ER-Ca^{2+} sensor regulating SOCE (20). However, the exact mechanism by which STIM1 regulates SOCE is not yet known. The second protein, Orai1, is a four transmembrane domain protein that was initially suggested to be a plasma membrane-localized regulatory protein for I_{CRAC}. Mutations in Orai1 have been genetically linked to SCID and T-lymphocytes isolated from SCID patients displayed decreased I_{CRAC} activity (23). Knockdown of Orai1 decreased SOCE, while expression of Orai1 with mutations in conserved negatively charged residues changed the Ca^{2+} selectivity of CRAC channel (24-27). Co-expression of Orai1 with STIM1 greatly enhanced CRAC channel activity (28-30). Since Orai1 and STIM1 can reconstitute the I_{CRAC} current, it has been suggested that they are sufficient for the formation of this channel.

We have previously reported that TRPC1 is an essential component of the SOC channels in salivary gland cells (13,16,31-33). Our data demonstrated that knockdown of TRPC1 decreases SOCE, overexpression increases the activity, while expression of TRPC1 with mutations in the proposed pore region of this protein alters the Ca^{2+} permeability of the channel. In the present study, we have investigated the possible role of STIM1 and Orai1 in TRPC1-dependent SOCE. The data presented here reveal that all three proteins are essential for generation of TRPC1-SOC channels. We show that STIM1 regulation of TRPC1-SOC is similar to its regulation of I_{CRAC}. Further, we report that dynamic assembly of a TRPC1/STIM1/Orai1 complex is involved in activation of Ca^{2+} entry. These data reveal common components of SOC and CRAC channels that contribute towards their store-dependent regulation and function.

**Experimental Procedures**

**Materials.** Fura-2AM and thapsigargin (Tg) were obtained from Calbiochem. (San Diego, CA, USA). Glass-bottomed 35mm petri dishes were obtained from MatTek Corporation (Ashland, MA, USA) and glass coverslips from Fischer Scientific (Pittsburgh, PA, USA). Anti-STIM1 antibody was obtained from BD Biosciences (San Jose, CA, USA), anti-myc antibody was from Cell Signalling (Danvers, MA, USA), anti-HA antibody from Roche (Indianapolis, IN, USA), and anti-FLAG antibody from Sigma Aldrich (St. Louis, MO, USA). Cy5- and FITC- and TRITC-tagged secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). STIM1 and Orai1 siRNAs were obtained from Dharmacon (Lafayette, CO, USA).

**Cell culture and transient transfection.** HSG, RBL-2H3, HEK-293, and A7r5 cells were cultered as described previously (6,22). Cells were transfected with the required vector as described previously (33) and used for experiments after 48h. Vectors encoding GFP were co-transfected (1:5 ratio of cDNA) to allow selection of cells for all functional experiments. Cells were transfected with siRNAs using the DharmaFECT transfection reagent as per manufacturer’s instructions and used after 48 hours. Sequences for the shRNA targeting human TRPC1 were similar to previously described siRNA sequence (15). The sense sequence (5’-CACCAGGTGACTTATATGGTTCGAAAACCATAATAGTCACCC-3’
and antisense sequence (5’-AAAAGGG-TGACTATTATATGGTTTTCGAACC ATATAATAGTCACCC-3’) was obtained from Integrated DNA Technologies (Coralville, IA, USA.) and hybridized in equal molar ratio and ligated into pENTR/U6 vector (Invitrogen, Carlsbad, CA, USA). 1 µg/ml TRPC1-shRNA (shTRPC1) plasmid was transfected into HSG cells. Construction of TRPC1 mutants has been reported earlier (16). For live cell imaging experiments, cells were plated on glass-bottomed MatTek tissue culture dishes. For patch clamp measurement and TIRFM, cells were plated on glass coverslips.

RNA isolation, synthesis of first-strand cDNA and RT-PCR analysis. RNA isolation, synthesis of first-strand cDNA and RT-PCR analysis were conducted using methods described previously (23,32). Sequences of primers (Integrated DNA Technologies) used to detect the Orai1 transcript are as follows: 5’-CAGCAGCCAACGTCAGCACCA-3’ and 3’-CAGCTGGTCCTGTAAGCGGGCAAA-5’. The -ve indicates the buffer control whereas +ve control was done with the human cDNA aliquot provided in the RT-PCR kit, FastStart High Fidelity PCR system from Roche Applied Sciences (Indianapolis, IN, USA). The PCR product was sequenced and confirmed to be the Orai1 transcript.

[Ca^{2+}]_i measurements. Measurements of [Ca^{2+}]_i were performed by imaging Fura-2 loaded cells using an Olympus 50x microscope (Olympus, Center Valley, PA, USA), with an ORCA-ER camera (Hamamatsu, Tokyo, Japan) attached to a Polychrome IV (Till Photonics LLC, Pleasanton, CA, USA). MetaFluor (Molecular Devices, Downingtown, PA, USA) was used to acquire images and processing the data. Other details have been provided elsewhere (33). In the case of transfected cells, GFP was used to select the transfected cells. Measurements were made in standard extracellular solution (SES): 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH), additions of Ca^{2+} are indicated (16). Relative changes in 340/380 ratios (representing [Ca^{2+}]_i) are shown in the figures (traces are average of at least 50-100 cells in each experiment).

Electrophysiological measurements. Whole cell-attached patch clamp measurement was performed using Axopatch 200B amplifier (Molecular Devices) as described previously (6,33). Patch pipette resistance was 3 to 6 mΩ filled with the following solution: 145 mM cesium methane-sulfonate, 8 mM NaCl, 10 mM MgCl_2, 10 mM HEPES, 10 mM EGTA, pH 7.2 (CsOH). SOCE was activated by either including IP_3 (10 µM) in the pipette solution or by including Tg (1 µM) in the external solution (as indicated in the figures). Standard external solution contained: 145 mM NaCl, 5 mM CsCl, 1 mM MgCl_2, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH), containing 10 mM CaCl_2. For Na^{+} current measurements the external solution (divalent cation free, DVF) was composed as follows: 165 mM NaCl, 5 mM CsCl, 10 mM EDTA, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH). Development of the current was assessed from the current amplitudes at potentials of −80 mV and +80 mV recorded during voltage ramps ranging from −90 to 90 mV (over a period of 1 s, imposed every 4 s) from a holding potential of 0 mV, and digitized at a rate of 1 kHz. A liquid-junction
potential of less than 8 mV was not corrected, capacitative currents and series resistance were determined and minimized. The first ramp was used for leak subtraction in the subsequent current records.

Western Blotting and Immunoprecipitation. Cells, grown to 80% confluence, were harvested and crude membrane fraction (used where indicated) was prepared as described previously (33,34). This preparation was solubilized in SDS-sample buffer and analysed by SDS-PAGE and Western Blotting (25-50 µg of protein were loaded per lane, details described in 33, 34). Where indicated, cells were treated with 1 µM thapsigargin or vehicle for 3 min in a Ca\(^{2+}\)-containing SES, lysed with RIPA buffer containing proteolytic inhibitors. The lysates were centrifuged at 50,000xg for 30 minutes and the supernatants were precleared and used for immunoprecipitation (IP) as described previously (33,34) using 1:100 or 1:200 dilution of antibodies. Immunoprecipitates were released by solubilization in SDS-sample and resolved by Western Blotting. Where indicated, band intensities were quantified in an image analyzer, Gel Doc™ XR, using the Quality One software (Bio-Rad, Hercules, CA, USA). Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses in this and other experiments were done using the Student's t-test.

Purification of GST-N- and GST-C-TRPC1 proteins and GST pull down assays. The N and C terminus of TRPC1 was cloned into pGEX5.1 (Amersham Biosciences, Piscataway, NJ, USA) vector and expressed as a GST fusion protein. GST pull down assays were conducted as described previously (33,35). Briefly, 500 ml of *Escherichia coli* (BL-21) expressing either GST or GST-N-TRPC1 or GST-C-TRPC1 protein were induced with 0.2 mM IPTG. Proteins were purified after lysing the cells by French press at 1200 pounds/square inch. Cell debris was removed by centrifugation at 10,000g for 10 min. The supernatant was loaded on a glutathione S-agarose affinity column, washed five times with PBST buffer, and eluted using 10 mM reduced glutathione. 2.2 µM of the individual GST purified proteins were incubated with 500 µg of HSG cell lysates (lysed either using RIPA buffer or octylglucoside + 500 mM KI containing proteolytic inhibitors, see #33, 34) for 60 min. Interacting proteins were pulled down using 100 µl of GST beads, washed five times, with high salt buffer and incubated with SDS sample buffer. Proteins were resolved on a 10% SDS-PAGE gel and Western blots were performed as described above.

Preparation of dispersed mouse submandibular gland cells. Cells were prepared from the mouse submandibular gland as described previously (31). Briefly, submandibular glands were removed, cleaned, minced, and digested in SES buffer containing 0.02% soybean trypsin inhibitor and 0.1% bovine serum albumin containing collagenase P (2.5 mg/8 ml) for 15-20 min at 37 °C, washed twice with the external solution and resuspended in 5 ml of SES buffer. Cell suspension was then treated with Tg for 60 s, centrifuged, and pellets were solubilized in RIPA buffer for 30 min at 4°C. Lysates were obtained after centrifugation, precleared and used for immunoprecipitation as above. All mice
were maintained according to guidelines approved by the NIDCR, National Institutes of Health, Animal Care and Use Committee.

**Immunofluorescence and confocal microscopy.** Cells were fixed and permeabilized as described previously (31,32) and then treated with the required antibody (indicated in each case) at 1:100 dilution. Samples were washed and then treated with Cy5- and FITC- or TRITC-tagged secondary antibodies as required (1:100 dilution). Images were collected using a Leica Confocal microscope. Images were analysed using MetaMorph software (Molecular Devices). Control experiments were done without primary antibody.

**TIRFM imaging.** TIRFM imaging was conducted using an Olympus IX81 motorized inverted microscope (Olympus). Excitation light was provided by a 20mW Argon Krypton laser. The 488nm laser was directed into an Olympus TIRF illuminator attached to the rear port of the microscope and through a 488 band pass filter (BP 10nm) to a TIRF-optimized Olympus Plan APO x60 (1.45NA) oil immersion objective. Emitted light was collected through a 525 band pass filter (BP 50nm). Images were collected every 0.5 s using a Hamamatsu EM C9100 back-thinned camera (Hamamatsu) controlled using the Openlab modular imaging software (Improvision, Lexington, MA, USA). Cells were bathed in a Ca\(^{2+}\)-containing standard extracellular solution (recipe as described above). Solution changes were accomplished by selecting flow from a multi-chambered valve controlled mechanical fed reservoir.

**Results**

**Effect of knockdown of TRPC1, Orai1, or STIM1 on SOCE and generation of \(I_{SOCE}\).** Knockdown of endogenous TRPC1, STIM1 or Orai1 using shTRPC1, STIM1 siRNA, or Orai1 siRNA reduced Tg-stimulated Ca\(^{2+}\) influx as compared to that in cells transfected with the relevant control vector or siRNA (Figures 1A-C, red and black traces respectively). Internal Ca\(^{2+}\) release was not altered. (Note that shTRPC1 was used since attempts to knockdown TRPC1 with several different siRNAs, either commercially available or custom made, were not very successful even after 72h of transfection). Consistent with the effect on Ca\(^{2+}\) influx, the store-operated current, \(I_{SOC}\), which we have previously described in HSG cells (6,16,36) was greatly attenuated in cells transfected with STIM1 siRNA (Figures 1D and E, red traces) as compared to the currents in cells transfected with non-targeting siRNA or in untransfected cells (black traces in the figures). shTRPC1 also reduced \(I_{SOC}\) (data not shown), the decrease was similar to what we have previously reported in cells treated with antisense TRPC1 (13,16) Endogenous STIM1 protein was detected in crude membrane preparations from RBL and HSG cells and rat brain (Figure 1F). STIM1 was detected as a doublet in RBL and HSG cells and as a single band in the brain samples. Notably, knockdown of protein expression in HSG cells using STIM1 siRNA (confirmed by Western blotting), showed reduction in both bands in siRNA-treated samples as compared to controls (Figure 1G). Whether the two bands represent different glycosylated states of STIM1 is not known. Orai1
transcript was detected in HSG cells by RT-PCR (Figure 1H, markers as well as positive and negative controls are shown) and expression of Orai1 siRNA significantly reduced I_{SOC}. Most cells (6/11) displayed a >90% inhibition of the current (Figure 1I). However, some Orai siRNA-treated cells (4/11) displayed transient I_{SOC} current, the amplitude of these transients decreased with time (Figure 1J). 1/11 cells displayed very little effect of Orai siRNA (data not shown). Figure 1K shows the current-voltage (I-V) relationship of the currents shown in I and J (maximum currents detected in I and J were used for plotting the I-V curves, black and red traces respectively). Together, these data suggest that TRPC1, STIM1, and Orai1 are required for SOCE and SOC channel function in HSG cells.

Effect of overexpression of TRPC1, Orai1, or STIM1 on SOCE and I_{SOC}. Similar to our previous findings, overexpression of TRPC1 induced about 2-fold increase in SOCE and I_{SOC} (Figures 2A–C). Overexpression of STIM1 (YFP-STIM1) or myc-Orai1 did not change Tg-stimulated Ca^{2+} release or significantly increase Ca^{2+} influx or I_{SOC} currents (Figures 2D–F and I–K, respectively). Further, STIM1 with mutations in the EF-hand domain (YFP-STIM1D76A) induced constitutive activation of SOCE. Expression of YFP-STIM1D76A in HSG cells increased basal Ca^{2+} entry to a level similar to that seen after Tg stimulation (Figure 2G, black trace). This entry was inhibited by 1 μM Gd^{3+} (Figure 2G, red trace, basal entry in control cells was similar to that seen in the presence of Gd^{3+}). Further, expression of this mutant of STIM1 did not change Tg-induced internal Ca^{2+} release (Figure 2H). Thus, as suggested for CRAC channels (24,25,29,37,38) SOC channel in HSG cells is activated by STIM1. The effect of store-depletion on STIM1 localization in HSG cells was also consistent with the pattern seen in other cell types (Supplemental Figure 1). YFP-STIM1 had a uniform distribution throughout the cells while the EF-hand mutant, YFP-STIM1D76A, was detected in the subplasma membrane region. Further, while YFP-STIM1 relocated to punctae in subplasma membrane region after Tg treatment, YFP-STIM1D76A localization was not changed by Tg. Additionally, expression of STIM1 C-terminus, but not STIM1 lacking the C-terminus, induced spontaneous activation of SOCE (activation was about 50% of maximum, data not shown), suggesting that STIM1 C-terminus is involved in regulation of SOCE (data not shown). Similar findings have been reported by Huang et al. (37). Importantly, Orai1 overexpression did not alter the magnitude or characteristics of Tg-stimulated Ca^{2+} entry or I_{SOC} in HSG cells in contrast to that seen in HEK293 cells where Orai1 expression decreased endogenous SOCE (27,30).

Effect of TRPC1 overexpression on I_{CRAC} in RBL cells. While Orai1 and STIM1 appear to be sufficient for generation of CRAC channels, the data presented above demonstrate that STIM1, Orai1, and TRPC1 are required for SOC channel function in HSG cells. We therefore assessed the effect of TRPC1 on CRAC channel activity in RBL cells which display a classical I_{CRAC} when intracellular Ca^{2+} stores are depleted (2,8). Compared to the characteristics of I_{CRAC} (shown in Figure
3A) transient expression of TRPC1 (48h after transfection) induced a left-shift in the reversal potential generating a current which was more $I_{\text{SOC}}$-like (compare traces in Figure 3B with those in A) in $\text{Ca}^{2+}$-containing ($\text{Ca}^{2+}$+$\text{Na}^{+}$) and divalent cation free (DVF) external medium. TRPC1 expression did not alter the magnitude of the currents in either condition (Figures 3C and D, also see Figures 3H and I for average change in reversal potential and current amplitudes). Thus, TRPC1 appears to modify $I_{\text{CRAC}}$. To further examine this, mutants of TRPC1, previously shown to alter SOC permeability in HSG cells (16) were expressed in RBL cells. Simultaneous substitutions of seven acidic aa in the S5-S6 region (Asp --> Asn and Glu --> Gln, mut pore) or single mutation, D581K, also altered cation permeability of CRAC (Figures 3E and F, respectively). Average reversal potentials measured in each case, $E_{\text{rev}}$, as well as current densities are shown in Figures 3H and I, respectively. Additionally, expression of TRPC1 truncated after the $5^{\text{th}}$ transmembrane (no-pore), induced dominant suppression and dramatic changes in $I_{\text{CRAC}}$ (Figures 3G, H, and I). Interestingly, and in contrast to its effect on $I_{\text{SOC}}$ in HSG cells, antisense TRPC1 did not affect $I_{\text{CRAC}}$ (trace not shown, see average data in Figures 3H and I) The latter finding agrees well with that of the lack of endogenously expressed TRPC1 in these cells (Figure 3J). Similar effects of TRPC1, mutants of TRPC1, or antisense-TRPC1 on store-operated calcium entry in RBL cells were also detected by Fura-2 fluorescence measurements (Figures 3K and L). In aggregate, the data shown in Figure 3 suggest that although TRPC1 is likely not an endogenous component of CRAC in RBL cells, it can interact with the core components of this channel CRAC and modify its properties. This is specially illustrated by the dominant negative effect induced by TRPC1 lacking the pore domain. Orai1 has been suggested to contribute to the pore of CRAC channels (24-26) although the effect of Orai1 siRNA on $I_{\text{CRAC}}$ in RBL cells has not yet been demonstrated. Thus, the data shown in Figure 3 demonstrate the potential of TRPC1 to interact with CRAC channel components. It is interesting to note that the effect of expression of Orai1 mutants on CRAC channel activity are very similar to the effects induced by expression of TRPC1-mutants on $I_{\text{CRAC}}$ (data in Figure 3) and $I_{\text{SOC}}$ reported earlier (16).

**Association of TRPC1, STIM1 and Orai1.** To examine the possible interaction of TRPC1, STIM1, and Orai1, we first determined their localization in HSG cells. Figure 4A shows co-localization of (i) STIM1 (green signal) and TRPC1 (red signal); (ii) STIM1 (green signal) and Orai1 (red signal); (iii) TRPC1 (red signal) and Orai1 (green signal, yellow color indicates overlap in the localization of the proteins in all the panels). STIM1 was diffusely localized in the cytosolic region of the cells (Figure 4A, i and ii), Orai1 was primarily detected in the plasma membrane region (Figure 4A, ii and iii). TRPC1 was found in the internal region of the cells as well as the plasma membrane (i and iii) and strongly co-localized with Orai1 (iii). Following stimulation with thapsigargin, there was increase in the co-localization of TRPC1 and STIM1 in the plasma membrane region (see right panel in i) as well as STIM1 and Orai1 (ii, right panel), but no further change in the co-localization of
TRPC1 and Orai1 (iii, right panel). Note that although STIM1 localization in thapsigargin-treated cells appears to be somewhat punctate (Figure 4A i and ii, right panels) this is more clearly detected using TIRF microscopy (see Supplemental Figure 1B). Possible interaction between TRPC1, STIM1, and Orai1 were further examined by immunoprecipitation experiments. The data in Figure 4B and C show association between TRPC1 and STIM1 in HEK293 and A7r5 cells, respectively (controls and transiently transfected with STIM1 cDNA). Both cell types endogenously express TRPC1 and furthermore TRPC1 has been shown to be involved in SOCE in both cases (9,15). Either anti-TRPC1 (HEK293 cells) or anti-STIM1 (A7r5 cells) was used for immunoprecipitation (IP) and proteins in the immunoprecipitated fraction were detected using either anti-STIM1 or TRPC1 antibodies (IB). In HEK293 cells, STIM1 was co-immunoprecipitated with TRPC1 from control and STIM1-overexpressing cells (upper panel, note there is more STIM1 in the latter). Lower panel shows the immunoprecipitation of TRPC1 which was similar in both sets of cells. Similarly, in A7r5 cells, endogenous TRPC1 was co-immunoprecipitated with STIM1 (Figures 4C, upper panel, anti-STIM1 antibody was used for the immunoprecipitation). Lower panel shows levels of STIM1 in the immunoprecipitates from both samples. Note that the amount of TRPC1 immunoprecipitated with STIM1 is determined by the levels of STIM1 expressed in the cells (more STIM1 and TRPC1 are seen in the IP fraction from cells overexpressing STIM1).

We also used GST fusion proteins of the N- and C-terminus of TRPC1 (GST-N-TRPC1 and GST-C-TRPC1, respectively) to further examine the STIM1-TRPC1 association. STIM1 from HSG cell lysates bound to GST-C-TRPC1, but not to either GST alone or GST-N-TRPC1 (Figure 4D, upper panel). Lower panel shows the amounts of GST-fusion proteins of N-TRPC1 and C-TRPC1 used.

**Effect of thapsigargin stimulation on TRPC1/STIM1/Orai1 complex.** Figure 5 shows the effect of thapsigargin on the interaction of TRPC1, STIM1, and Orai1 in salivary gland cells. Interaction of endogenous STIM1 (indicated “*”) or overexpressed YFP-STIM1 (indicated “**”) with endogenous TRPC1 increased following stimulation of HSG cells with 1 µM Tg (Figures 5A and B show results of IP with anti-TRPC1 antibody and IB with anti-STIM1). Note that expression of YFP-STIM1 also increased the association of endogenous STIM1 with TRPC1 (Figure 5B) likely due to heteromerization between the exogenous and endogenous STIM1 proteins. The increase in STIM1-TRPC1 interaction following Tg stimulation was also seen in acutely dispersed mouse submandibular gland cells (Figure 5C). Additionally, exogenously expressed myc-Orai1 interacted with endogenous TRPC1 as well as endogenous STIM1 in HSG cells. More importantly, the association of TRPC1 and STIM1 with myc-Orai1 increased following Tg treatment (Figure 5D, left panel shows IP with anti-myc; 1.5 ± 0.18 and 1.36±0.1-fold respectively, n=3). In the reverse experiments, IP of endogenous TRPC1 (Figure 5D, right panel) pulled down myc-Orai1 which was increased
(1.8±0.22-fold, n=3) when cells were stimulated with Tg. Endogenous STIM1 was also increased in the IP fraction (1.4±0.1-fold, n=3) suggesting that STIM1 continued to interact with TRPC1 in these myc-Orai1-expressing cells and that there is no apparent competition between Orai1 and TRPC1 for STIM1. Tg-induced increases in the levels of TRPC1 and STIM1 with myc-Orai1 and of myc-Orai1 and STIM1 with TRPC1 were similar (based on the quantitation of the blots shown in Figure 5D). These important data demonstrate that internal store depletion promotes association between TRPC1, Orai1, and STIM1.

Discussion

Our previous studies have established that TRPC1 determines the cation permeability of SOC channel and thus is required for SOCE in salivary gland cells (16). Here we report that that Orai1 and STIM1 contribute to TRPC1-dependent SOC channel function. Orai1 and Stim1 have garnered much attention recently as the elusive molecular components of CRAC channels based on the observation that co-expression of the two proteins is sufficient for generation of I_{CRAC} (23-25,28-30,38). The data presented above demonstrate that in addition to TRPC1, Orai1 and STIM1 are also required for SOCE and SOC channel activity in HSG cells. Consistent with recent reports (39) we show that STIM1 is the ER-Ca^{2+} sensor involved in regulation of SOCE in HSG cells. Knockdown of STIM1 induced a dramatic inhibition (>90%) of I_{SOC} in HSG cells while expression of a STIM1 EF-hand mutant, which lacks Ca^{2+} sensitivity, generated spontaneous SOCE. Further, STIM1 relocated in the subplasma membrane region of HSG cells in response to Tg-induced internal Ca^{2+} store depletion. Thus, regulation of STIM1 by internal Ca^{2+} store depletion is similar in cells expressing I_{CRAC} and those with TRPC1-dependent I_{SOC}. In aggregate, these findings demonstrate that STIM1 in the ER is likely to be an upstream channel regulator of both SOC and CRAC channels. The more significant, and somewhat unexpected, finding of our study is that Orai1 also contributes to TRPC1-SOC channel function. We have shown that knockdown of Orai1 reduced (by >90%) I_{SOC}. Importantly, we show that internal Ca^{2+} store depletion induced dynamic assembly of a TRPC1/STIM1/Orai1 ternary complex. Together, these novel findings suggest that TRPC1, Orai1, and STIM1 concertedly determine SOCE and contribute to SOC channel activity in HSG cells. Thus, these data suggest similarities in the molecular components of CRAC and SOC channels. It will be important to determine whether this is true for different SOC channels that have been identified in various cell types and especially those involving TRPC1.

Reports demonstrating that Orai1 and STIM1 are essential components of SOCE (as discussed above) rule out the contribution of TRPCs to SOCE based on lack of effect of siRNAs (20,21,25) as well as lack of mutations in TRPC proteins in T lymphocytes from SCID patients (20,21,25). However, as noted above TRPC channels have been suggested SOCE components in various cell types (2-4,10,11). Our previous studies have clearly established TRPC1 as a pore-forming component of SOC channel in HSG and other salivary gland cells (10,13,34). It should be also noted that while our present study indicate that
siRNA might not be very effective in reducing TRPC1 in HSG cells even after treatment for 24 or 48 h, shRNA as well as antisense RNA were very effective in reducing TRPC1 levels which was accompanied by a decrease in function. Notably, several earlier reports have implicated a role for TRPCs in SOCE in the same cell types that were used for the Orai1 and STIM1 studies e.g. TRPC3 in T-lymphocytes (18), TRPC1, TRPC3 and TRPC7 in HEK293 cells (15) and TRPC1 in DT-40 cells (14). In this context, it is important to emphasize that overexpressed STIM1 or Orai1 can interact with endogenous TRPC1 (see Figure 5) and further, overexpressed TRPC1 can modify CRAC channel activity, likely by interacting with the channel components (Figure 3). An association between STIM1 and TRPC1 has been convincingly demonstrated in HEK293 cells and platelets (37,40). Further, Huang et al. (37) have shown that STIM1 selectively interacts with TRPC proteins, TRPC1, TRPC2, and TRPC4 interacted with STIM1 while TRPC3, TRPC6, and TRPC7 did not. Whether this difference is related to the ability of these TRPCs to form SOCs is not yet known. Together, these findings provide evidence for an association between TRPC1 and the CRAC channel components, Orai1 and STIM1. The present findings not only substantiate previous suggestions made by us and others that TRPC channels function as molecular components of SOC channels (2-4,10,11) but also demonstrate for the first time that TRPC1, Orai1, and STIM1 are concertedly involved in SOCE.

Although we have not directly assessed whether Orai1 contributes to the Ca\(^{2+}\) permeability of SOC, based on recent reports suggesting that it is a pore-forming subunit of CRAC channels, it is possible that it also contributes to SOC channel permeability in HSG cells. While exactly how two potentially distinct pore-forming proteins, i.e. TRPC1 and Orai1, contribute to SOC channel activity is presently not clear, our data identify a requirement for both TRPC1 and Orai1 in SOCE and generation of I\(_{SOC}\) in HSG cells. As discussed in a recent review by Smyth et al. (39), TRPC1/STIM1 and Orai1/STIM1 could function as distinct channels and contribute independently to SOCE. Our data argue against the suggestion that Orai1 and STIM1 generate an independent CRAC channel in HSG cells. Knockdown or overexpression of Orai1 or TRPC1 does not change the characteristics of the I\(_{SOC}\) in HSG cells. If TRPC1/STIM1 and Orai1/STIM1 formed independent channels in HSG cells, knockdown of TRPC1 would have generated an I\(_{CRAC}\)-like current due to the residual Orai1/STIM1-CRAC channel activity and knockdown of Orai1 would have decreased the contribution of I\(_{CRAC}\) to the total currents. Further, as discussed above, TRPC1 has the potential to interact with core components of the CRAC channel. Thus we have proposed that TRPC1, Orai1, and STIM1 together form the functional SOC channel in HSG cells (see model in Figure 6). However, further studies will be required to determine the exact molecular composition of this channel and how it is regulated by internal Ca\(^{2+}\) store depletion.

The currently accepted concept for activation of CRAC channels is that internal Ca\(^{2+}\) store depletion induces relocation of STIM1 into aggregates in ER. Formation of STIM1 aggregates in
the subplasma membrane regions, and its likely interaction with Orai1 in the plasma membrane, appear to be prerequisite for activation of the channel (25,28,30,38,39,41). While an increase in co-localization of these two proteins in the subplasma membrane region has been shown in several cell types, there are no data to directly demonstrate an interaction between the two proteins. We and others have previously suggested that regulation of TRPC1-dependent SOC channels is determined within functionally specific microdomains in the cells (10,42). Both ER and plasma membrane components are involved in channel regulation and this is achieved by the close apposition of the intracellular membrane with the surface membrane. Consistent with this suggestion TRPC1 and STIM1 were shown to redistribute into the same punctae locations in the subplasma membrane region in response to internal Ca\textsuperscript{2+} store depletion (37). Further, an increase in endogenous STIM1-TRPC1 interaction following thapsigargin treatment was shown in platelets (40) although no increase in protein association was detected with the overexpressed proteins in HEK293 cells (37). Here we have shown that upon internal Ca\textsuperscript{2+} store depletion STIM1 relocates into punctae in the ER which co-localize with Orai1 and TRPC1 in the plasma membrane region. Further, there is increased association of STIM1 with Orai1 and TRPC1 in stimulated cells. Thus, we suggest that STIM1 in junctional ER associates with TRPC1 and Orai1 in specific plasma membrane microdomains during activation of SOCE.

In summary, the data we have described above demonstrate that that TRPC1 can associate with two critical components that have been shown to regulate/generate I\textsubscript{CRAC}, Orai1 and STIM1. Further, we have shown that all three proteins are required for SOCE and SOC channel function. Together, our data provide evidence for similarities in the molecular components of SOC and CRAC channels. A major finding in this study is that dynamic assembly of a TRPC1/STIM1/Orai1 ternary complex is associated with activation of SOCE. An important question that needs to be addressed is how Orai1, STIM1 and TRPC1 assemble to form SOC channel(s). Our previous studies, and those of several other groups have demonstrated that TRPC1 is assembled in a macromolecular signaling complex along with key Ca\textsuperscript{2+} signaling proteins, e.g. SERCA, PMCA, PLC\textbeta, IP\textsubscript{3}R, and calmodulin. In addition roles for Homer (43), Rho-GTase (44), caveolin-1 as well as plasma membrane lipid raft domains (34,45) have been suggested in the regulation of TRPC-associated SOCE. It is interesting that IP\textsubscript{3}Rs have also been recently shown to relocate into punctae in the subplasma region of cells (46). Further studies will be required to resolve the exact molecular rearrangements that govern the interactions between TRPC1, Orai1, and STIM1 and to define the specific individual functions of the accessory proteins involved in SOCE.

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Figure 1. Effect of knockdown of TRPC1, Orai1, or STIM1 on SOC channel function. Cells were transfected with (A) shTRPC1, (B) STIM1 siRNA or (C) Orai1 siRNA and thapsigargin (Tg)-stimulated SOCE was measured 48h after transfection. [Ca^{2+}]_i was measured in Fura-2 loaded cells and is expressed as 340/380 ratio (cells transiently transfected with control vector, black traces, and sh- or siRNA vectors, red traces). (D) Current traces in this and subsequent figures represent the amplitude at -80 mV (inward current) and 80 mV (outward current) recorded during voltage ramps (1 s between -90 mV to 90mV) that were applied every 4 s as described in Experimental Procedures. (E) I-V relationship of macroscopic currents induced by Tg in control cells and STIM1 siRNA transfected cells (trace shown is the maximum current recorded in D). (F) Western blot showing expression of STIM1 protein (indicated by arrow) in crude membrane fractions isolated from RBL and HSG cells as well as in rat brain (25 µg of proteins were loaded in each lane, note that all three were run on the same gel). (G) Western blot showing knockdown of endogenous STIM1 protein in HSG cells treated with STIM1 siRNA. “Con” indicates cells treated with non-targeting siRNA. (H) RT-PCR detection of Orai1 expression in HSG cell (-ve control indicates sample with no cDNA, +ve control was done with the human cDNA aliquot provided in the RT-PCR Kit (see Experimental Procedures). (I, J) Tg-induced I_{SOC} measured in cells transfected with Orai1 siRNA. I shows pattern obtained in 6/11 cells while J shows pattern (transient currents) obtained in 4/11 cells. 1/11 cells did not display any effect of Orai1 siRNA. (K) I-V relationship of the currents shown in I and J (black and red traces respectively, red trace shows the maximum current measured in J).

Figure 2. Effect of overexpression of TRPC1, STIM1, and Orai1 on SOC channel function. Cells were transfected with the respective vectors encoding TRPC1, STIM1, or Orai1 (red traces) or the control vectors (black trace) for 48 h after which they were used for Fura-2 or whole cell patch clamp measurements. [Ca^{2+}]_i measurements (A) and I_{SOC} (B, C) were measured in control HSG cells and cells overexpressing TRPC1. [Ca^{2+}]_i measurements (D) and I_{SOC} (E,F) in cells overexpressing YFP-STIM1. Basal Ca^{2+} entry was not altered by TRPC1 or STIM1 overexpression (data not shown). (G) Effect of YFP-STIM1D76A expression on basal Ca^{2+} entry. Ca^{2+} was added where indicated in both black and red traces. 1 mM Gd^{3+} was added to cells prior to Ca^{2+} addition in the red trace. Basal entry in control cells, not shown, was similar to that seen in the presence of 1 mM Gd^{3+}. (H) Effect of YFP-STIM1D76A expression on Tg-stimulated internal Ca^{2+} release. 1 µM Tg was added to control (black) and transfected cells (red) bathed in Ca^{2+}-free medium. All other additions are shown in the Figure. Effect of Orai1 expression on Tg-stimulated [Ca^{2+}]_i increases (I) and I_{SOC} (J, K). Each analog plot showing Ca^{2+} release and Ca^{2+} entry is representative of at least 4 experiments, with each trace showing the average for at least 50 cells. Plots showing channel events and I-V relationships are representative of at least 3-4 experiments (10-15 cells) with each trace showing the plot obtained with a single representative cell.

Figure 3. Effect of overexpressing TRPC1 and its mutants on CRAC activity in RBL cells. (A-D) I_{CRAC} was measured in control RBL cells (A) and RBL cells
transiently expressing TRPC1 (B-D) in Ca\(^{2+}\)+Na\(^{+}\) medium (Ca\(^{2+}\) current, red traces) or divalent cation free (DVF) medium (Na\(^{+}\) current, black traces). A and B show I-V curves while C shows a time course of the current. Average data of recorded current densities are shown in D. (E-G) I-V relationship of I\(_{CRAC}\) measured in cells expressing TRPC1 mutants TRPC1-mut-pore in which all negatively charged residues in the S5-S6 region were simultaneously mutated, Asp to Asn and Glu to Gln (E), TRPC1-D581K (F), or TRPC1-no-pore in which the protein was truncated after the 5\(^{th}\) transmembrane (G). (H, I) Average data for reversal potential and current densities obtained in control RBL cells and RBL cells overexpressing TRPC1 and its mutants. Effect of antisense TRPC1 (TRPC1-as) on reversal potential and current density is shown in H and I (current trace not shown). Values marked “**” are significantly different from unmarked values (P<0.025; n\(\geq\)6 cells in each case; Students t-test). (J) Western blot showing TRPC1 expression in HSG cells but not in RBL cells (crude membrane preparations, 25 \(\mu\)g protein, from the respective cells were used for this blot, anti-TRPC1 antibody was used to detect endogenous TRPC1). (K) Effect of TRPC1 and mutant TRPC1 proteins (described above) on thapsigargin (Tg)-stimulated internal Ca\(^{2+}\) entry. K shows typical changes in [Ca\(^{2+}\)], in control RBL cells. (L) Average data of relative Ca\(^{2+}\) entry, i.e. [Ca\(^{2+}\)] increase seen after readdition of 1 mM Ca\(^{2+}\) to Tg-treated cells. Tg-induced Ca\(^{2+}\) release was not altered in these experiments. Values marked “**” are significantly different from unmarked values (P<0.025; n\(\geq\)6 cells in each case; Students t-test). Color key for the histograms is given in I.

Figure 4. Co-localization of TRPC1, Orai1, and STIM1 in HSG cells. (A) Localization of (i) TRPC1 and STIM1 (red, FLAG-TRPC1; green, CFP-STIM1; yellow; overlap of the two signals); (ii) Orai1 and STIM1 (red, myc-Orai1; green, YFP-STIM1; yellow, overlap) in resting and thapsigargin (Tg)-stimulated (3 min) HSG cells. Confocal imaging was used to detect protein localization (antibody concentrations and other details are given in Experimental Procedures. TRPC1 was overexpressed since the signal of the endogenous protein was relatively lower that those of expressed YFP STIM1 or myc-Orai1, note that similar findings were obtained with HA-tagged or FLAG-tagged TRPC1 and CFP- or YFP-STIM1). (B) Co-immunoprecipitation of STIM1 (upper panel) with endogenous TRPC1 (lower panel) from lysates of HEK293 cells. (C) Co-immunoprecipitation of TRPC1 (upper panel) with STIM1 (lower panel) from lysates of A7r5 cells. In each case cells were either transfected with empty vector (control) or with STIM1-encoding vector (STIM1). “Inp” indicates input levels of proteins in lysate and “IP” indicates immunoprecipitated proteins. TRPC1 in the IP fraction was not changed by Tg stimulation (data not shown). Antibodies for immunoprecipitation (IP) and immunoblotting (IB) are indicated in the figures. 2 mg of cell lysates were used for IP. (D) Pull down of STIM1 from HSG cells lysates by GST-fusion protein of the C-terminus and N-terminus of TRPC1 (GST-C-TRPC1 and GST-N-TRPC1, respectively) is shown in the upper blot. “GST” indicates the control GST protein alone. Lower blots show the amount of the fusion proteins or control GST protein used.

Figure 5. Effect of thapsigargin stimulation on TRPC1/STIM1/Orai1 interaction.
(A-B) Co-immunoprecipitation of TRPC1 with endogenous STIM1, YFP-STIM1, respectively from lysates of unstimulated (-) and stimulated cells (+) (3 min with 1 µM thapsigargin (Tg)). YFP-STIM1 (**) is detected as a band above the endogenous STIM1 (*). IP and IB antibodies are indicated. (C) Co-immunoprecipitation of endogenous STIM1 with TRPC1 from mouse salivary gland cell preparations (see Experimental Procedures for details). “IP” indicates immunoprecipitated proteins, input shows protein in the lysate. (D). Association of TRPC1 with STIM1 and myc-Orai1 in unstimulated and Tg-stimulated HSG cells transfected with myc-Orai1. Left panel shows proteins in IP using anti-myc. Right panel shows proteins in IP of TRPC1 using anti-TRPC1 from the same cell lysate. Input levels of proteins and IB antibodies are shown in the blot.

**Figure 6. Model depicting proposed interactions of TRPC1, STIM1, and Orai1 in unstimulated and stimulated cells.** Our model predicts that TRPC1 and Orai1 are likely in the plasma membrane while STIM1 is in the ER. Internal Ca$^{2+}$ store depletion induces a change in Ca$^{2+}$-binding to STIM1 in the ER lumen. This leads to aggregation of the protein and by an as yet unrecognized mechanism transmits a signal to Orai1 and TRPC1 which results in recruitment of the proteins into a ternary complex and activation of SOC channel.
FIGURE 1

A - D: Graphs showing the effects of various treatments on cell calcium levels, with STIM1 siRNA, control, and shTRPC1 treatments indicated.

E: Graph showing the effect of STIM1 siRNA on calcium levels at different voltages.

F: Western blots showing STIM1 expression in RBL, HSG, and rat brain.

G: Western blots showing STIM1 expression with Con siRNA.

H: Western blots showing orai1 expression in various conditions.

I - J: Graphs showing the effect of Orai1 siRNA on calcium levels under different conditions.

K: Graph showing the effect of Orai1 siRNA on calcium levels at different voltages.
**FIGURE 2**

**HSG + TRPC1**

- **A**: Graph showing the effect of Tg (1 µM) on Ca²⁺(mM) levels with HSG + TRPC1.
- **B**: Graph showing the effect of Tg (1 µM) on HSG and HSG + TRPC1.
- **C**: Graph showing current-voltage (I-V) relationship with HSG + TRPC1.

**HSG + STIM1**

- **D**: Graph showing the effect of Tg (1 µM) on Ca²⁺(mM) levels with HSG + STIM1.
- **E**: Graph showing the effect of Tg (1 µM) on YFP and YFP-STIM1.
- **F**: Graph showing current-voltage (I-V) relationship with HSG + STIM1.

**HSG + STIM1D76A**

- **G**: Graph showing the effect of Tg (1 µM) on Ca²⁺(1 mM) levels with STIM1 and STIM1D76A.
- **H**: Graph showing the effect of Tg (1 µM) on Ca²⁺(1 mM) levels with +Gd³⁺(1 µM) addition.

**HSG + Orai1**

- **I**: Graph showing the effect of Tg (1 µM) on Ca²⁺(mM) levels with Orai1 and Basal.
- **J**: Graph showing the effect of Tg (1 µM) on Orai1 Basal. Control and Control Basal.
- **K**: Graph showing current-voltage (I-V) relationship with Orai1 and Basal.
FIGURE 4

A

i

TRPC1
+ STIM1

color
Tg

ii

Orai1
+ STIM1

iii

Orai1
+ TRPC1

B

HEK293 Cells

C

A7r5 Cells

D

GST
GST-TRPC1
GST-C-TRPC1

IB : STIM1
IB : GST
FIGURE 5

A Endogenous STIM1

IB: STIM1

IB: TRPC1

IP: anti-TRPC1

B YFP-STIM1

IB: STIM1

IP: anti-TRPC1

IP: anti-TRPC1

C Mouse salivary gland

IB: TRPC1; IB: STIM1

D IP: anti-myc

IB: anti-TRPC1

anti-STIM1

anti-myc

input

IP
